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RESEARCH ARTICLE

AN APPROACH TO QUALITY CONTROL OF COCKROACH ALLERGEN EXTRACTS IN INDIA USING ELECTROPHORESIS AND BIOCHEMICAL METHODS

***,[1]Achla Prasad, [1]Sanjay Mendiratta, [1]Saurabh Jaiswal, [1]Preeti Sharma, [1]Surinder Singh and [2]Mahendra Kumar Agarwal**

[1]National Institute of Biologicals, MOH & FW, Govt. of India, A-32, Sec.-62, Institutional Area, NOIDA-201309, UP, India
[2]Respiratory Allergy and Immunology, Metro Centre for Respiratory Diseases, Metro Hospital, NOIDA, India

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ABSTRACT

The high prevalence of patients with asthma caused by cockroach allergens has been reported around the world as well as in India (30%). The crude non-standardized cockroach extracts are still used for diagnosis and treatment. Commercial cockroach allergen extracts are reported to vary in protein content and electrophoretic banding patterns indicating to batch to batch variations and pose a challenge to the clinicians for accurate diagnosis and efficacious immunotherapy (Gaur *et al*., 2009; Burastero 2011; Özdemir 2014). The batch to batch variation in cockroach extracts may be minimized by an appropriate dialysis method and using electrophoresis and biochemical techniques for quality control.

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INTRODUCTION

Batch to batch inconsistency and non-standardized commercial allergenic extracts used in India and abroad for diagnosis and immunotherapy compromise accuracy of *in-vivo* diagnosis by skin prick tests and effective allergen immunotherapy (Gaur *et al*., 2009; Burastero 2011; Özdemir 2014). There is a need to upgrade the quality control of allergen extracts and develop allergen certification center in India similar to Food and Drug Administration (FDA) and Centre for Biologics Evaluation and Research, (CBER) USA (Gaur *et al*., 2009). The only Monograph on Allergens is available in European Pharmacopoeia. The reference standards (national or international) are also not available for cockroach. Cockroach allergens induce IgE sensitization, and exposure to their high levels in the home is a major risk factor for asthma (Aruda *et al*., 2001, Tandon *et al*., 1990). In view of above, we carried out the present work on quality control of cockroach (*Periplaneta americana*) allergen extract at National Institute of Biologicals, India in collaboration with Metro Hospital, NOIDA.

***Corresponding author: Achla Prasad**
National Institute of Biologicals, MOH & FW, Govt. of India, A-32, Sec.-62, Institutional Area, NOIDA-201309, UP, India.

MATERIALS AND METHODS

Preparation and Biochemical analysis of Cockroach (P. americana) Extract

A total of 16 extracts of *P. americana* (E) were prepared, each from 1gm of lyophilized whole body powder- WBP, (Chaudhary, 1988).The lyophilized WBP was procured from commercial source (M/s All Cure Pharma Pvt. Ltd., New Delhi). The extraction involved overnight defatting at 4°C in di-ethyl ether, followed by overnight precipitation in ammonium bicarbonate (0.1 M, pH-7.8) at 4°C. The supernatant was collected by centrifugation (5,000 rpm, at 4°C for 15 minutes) and dialyzed by two different methods at 4°C; 1) E_{DT}: using dialysis tubing Spectra/ Por 3 membrane; MW cut off 3.5 kDa ; (n=8). 2) E_{CF}: dialysis by centrifugal filtration using Amicon Ultra–Ultracel-3K filtration unit

(Merck Millipore Ltd.) with a cut off of 3kDa (n= 8).The dialysates were freeze dried and stored at 4°C and henceforth referred as Whole Body Extracts (WBE).

The determination of protein content (µg / mg WBE) in each of 16 extracts was done by Modified Lowry's method. Bovine Serum Albumin (BSA, Sigma: A7030) was used as standard at 10, 20,30,40,60 µg/ml concentration (Lowry *et al*., 1951). Carbohydrate content was determined for each extract by Anthrone method using glucose as standard (Sigma, G5767) at 10,20,30,40 and 50 µg/ml concentration (Scott and Melvin, 1953). To validate the results, both protein and carbohydrate determinations were performed at least thrice for each of 16 extracts. Total recovery of protein and carbohydrate in 16 WBEs each obtained from 1 gram WBP were calculated.

The protein profile was analyzed by Sodium Dodecyl Sulphate Poly Acrylamide Gel-Electrophoresis (SDS-PAGE) under reducing conditions using 10% resolving gel (40 µg/per well). Bands were viewed after staining with Coomassie Brilliant Blue (Sigma B0149-5G) (Laemmli *et al*., 1970). The SDS-PAGE was run thrice for each extract to validate the reproducibility.

Statistical Tests

The mean values of yield, protein and carbohydrate contents for two dialysis methods were compared using Student's t-test / Wilcoxon Rank Sum tests. The t- Test was also used to compare the variations in two dialysis methods.

RESULTS AND DISCUSSION

The results of average yield of WBE (mg)/gm of cockroach WBP obtained by two dialysis methods (E_{DT} and E_{CF}), their protein and carbohydrate contents and total recovery in 16 WBEs obtained from 1 gram WBP are summarized in Table 1.

Yield of WBE (mg) / gm WBP in E_{DT} and E_{CT} Extracts

It is observed that total yield of WBE from one gram of cockroach WBP was significantly higher and more consistent when dialyzed by centrifugal filtration (E_{CF}) as compared to E_{DT} extracts dialysed conventionally by tubing method ($p \leq 0.04$) (Fig 1: a).

Protein content and total protein recovery in E_{DT} and E_{CT} Extracts

The protein content (µg / mg WBE) as well as total protein recovery in WBEs obtained from 1 gram WBP was higher and more consistent in E_{CF} than in E_{DT} extracts (Table 1, Fig. 1:b1 & b2). Though the protein content (µg / mg WBE) did not differ significantly, the difference was highly significant for the total protein recovery in WBEs ($p \leq 0.003$) using the two dialysis methods.

Carbohydrate contents and total carbohydrate recovery in E_{DT} and E_{CT} Extracts

Similar observations were made for carbohydrate contents in E_{CF} and E_{DT} cockroach extracts prepared by two dialysis methods. The difference was highly significant for the total carbohydrate recovery in WBEs ($p \leq 0.01$) obtained using two methods (Fig.1. c).

The precision of protein and carbohydrate estimations is substantiated by reproducibility of cumulative data obtained for BSA and glucose standards at each concentration used for protein and carbohydrate estimations respectively. The variations observed for total yield, protein and carbohydrate contents in E_{DT} prepared using dialysis tubing may be because of variations likely to occur in volume, pore size and temperature during longer duration required to complete (48-72 hrs.) as compared to dialysis by centrifugal filtration.

Table 1. Summary of Biochemical Analysis Performed for various extracts (n=16) prepared using two methods of dialysis

S.No.	Method of Dialysis	Yield (WBEmg/gm WBP)	Protein content (µg/mg WBE)	Total Protein recovered in WBE(mg/gm WBP)	Carbohydrate content (µg/mg WBE)	Total Carbohydrate recovered in WBE (mg/gm WBP)
E_{DT1}	Dialysis by Tubing	135.00	266.49	35.98	Not done	Not done
E_{DT2}	Dialysis by Tubing	80.00	465.67	37.25	Not done	Not done
E_{DT3}	Dialysis by Tubing	98.00	470.00	46.06	38.70	3.80
E_{DT4}	Dialysis by Tubing	112.90	437.19	51.61	28.40	3.06
E_{DT5}	Dialysis by Tubing	100.00	414.00	41.42	18.10	2.37
E_{DT6}	Dialysis by Tubing	58.14	584.50	33.98	16.40	0.99
E_{DT7}	Dialysis by Tubing	138.00	530.80	73.26	Not done	Not done
E_{DT8}	Dialysis by Tubing	108.00	486.30	52.08	27.45	2.72
E_{CF1}	Centrifugal filtration	122.50	532.33	63.62	34.11	4.04
E_{CF2}	Centrifugal filtration	122.60	517.00	61.79	32.68	3.77
E_{CF3}	Centrifugal filtration	130.26	500.30	63.15	30.78	3.56
E_{CF4}	Centrifugal filtration	135.40	490.00	66.87	26.83	3.70
E_{CF5}	Centrifugal filtration	132.00	530.00	70.44	29.43	3.91
E_{CF6}	Centrifugal filtration	124.00	515.00	65.16	31.15	3.82
E_{CF7}	Centrifugal filtration	135.00	495.00	66.58	31.93	4.27
E_{CF8}	Centrifugal filtration	142.50	504.00	71.92	30.20	4.31
	Mean Values ±SD	E_{DT} = 103.8.1±26.6 E_{CF} = 130.5±7.2	E_{DT} = 458.1±93 E_{CF} = 507.5±17.2	E_{DT} = 46.5±12.8 E_{CF} = 66.2±3.5	E_{DT} = 25.8±7.9 E_{CF} = 30.9±1.9	E_{DT} = 2.58±1.0 E_{CF} = 3.92±0.26
	p-value	$p \leq 0.04$	$p \geq 0.07$	$p \leq 0.00$	$p \geq 0.08$	$p \leq 0.01$

E_{CF}: Extract prepared by dialysis-centrifugal filtration, E_{DT}: Extract prepared by dialysis by tubing

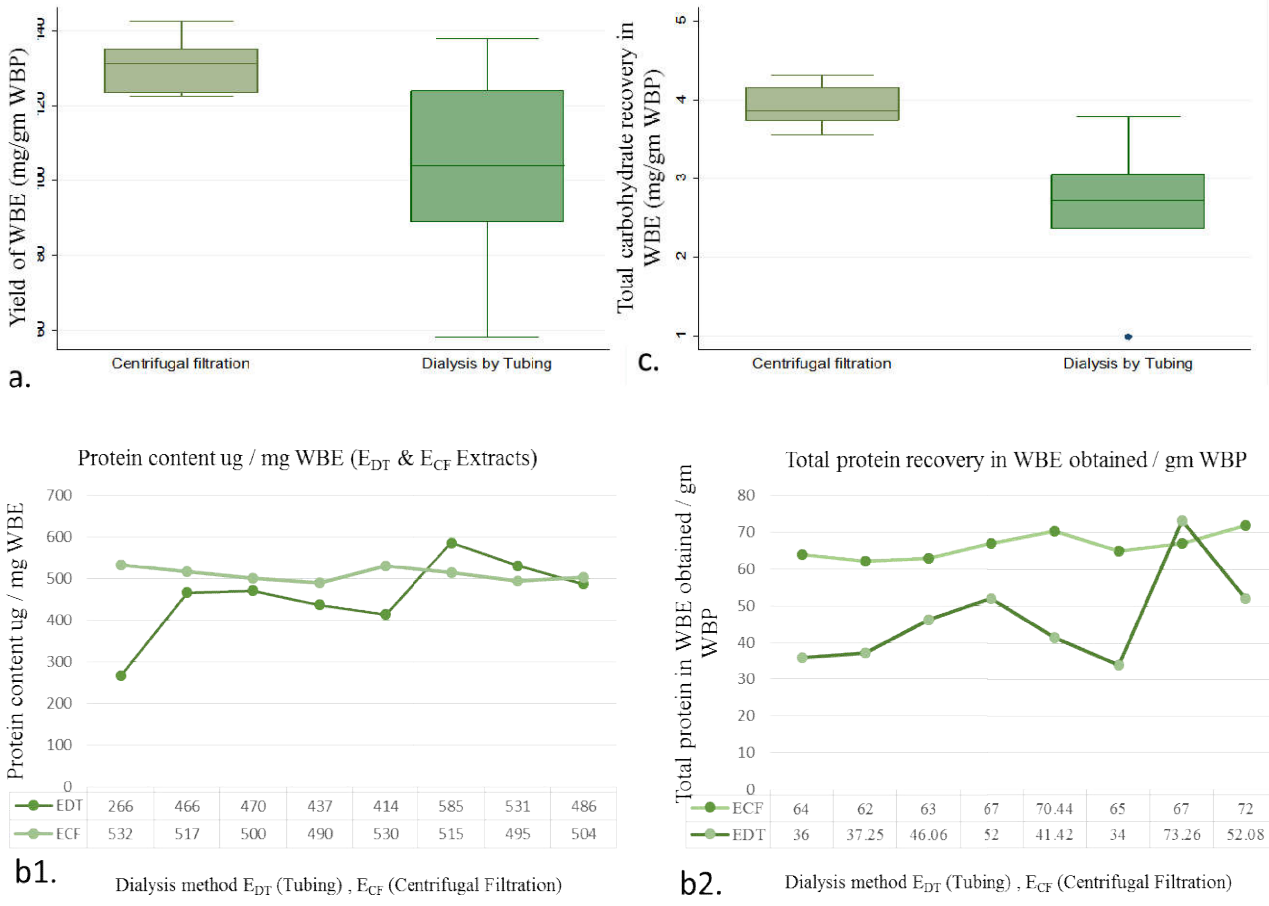

Figure 1. Biochemical Analysis of Whole Body Extracts (WBE) of Cockroach (*P. Americana*) prepared by dialysis using tubing (E_{DT}; n=8) and centrifugal filtration (E_{CF}; n=8) - Yield of WBE (mg) / gm of Cockroach Whole Body Powder (WBP) (a), Protein content and total protein recovery (b1 & b2) and Carbohydrate estimation (c) in 16 WBEs (E_{CF} and E_{DT}). There was significant difference in E_{CF} and E_{DT} in the yield of WBE ($p \leq 0.04$); total protein and carbohydrate recovery in WBE ($p \leq 0.003$ & $p \leq 0.012$ respectively)

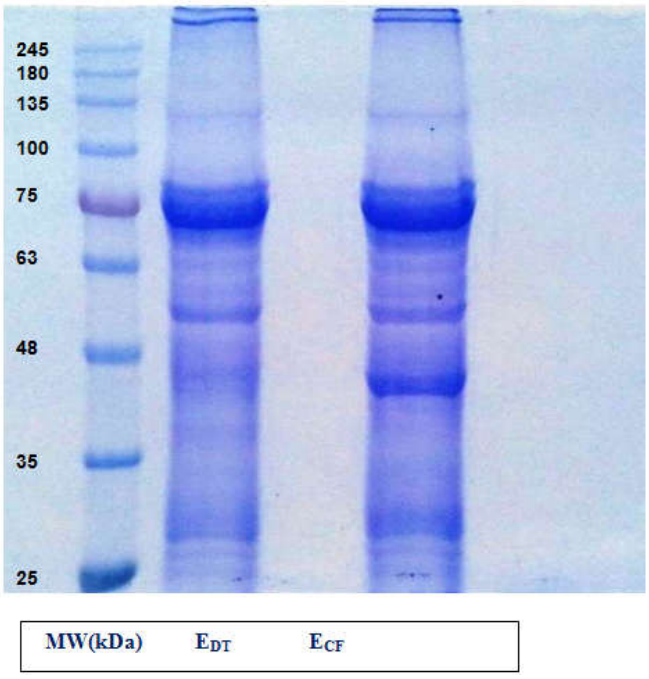

Figure 2. SDS-PAGE of Cockroach Extracts Prepared by Dialysis with Tubing (E_{DT}) and Centrifugal Filtration (E_{CF}); 40 µg of protein was loaded in each well

The centrifugal filtration method offers controlled parameters i.e. fixed pore size and volume, and is completed in shorter duration (in < 6 hours) under temperature maintained at 4°C during centrifugation.

It is reported that a 4-8 hour extraction in ammonium bicarbonate buffer (pH-8.0) yields extracts of optimal allergenic potency (Gaur *et al*., 2009). Also, the selection of raw material and extraction procedure, reflecting physiologic conditions of airways, are crucial for the preparation of an allergen extract (Jeong *et al*., 2011).

Variations in protein and allergen contents in allergen extracts affect both diagnosis and the administration of efficacious immunotherapy. Hence, availability of standardized and characterized allergen extracts is of prime importance for better patient care (Caldas *et al*., 2009; Tungtrongchitr *et al*., 2012). In present work, both E_{DT} and E_{CF} cockroach extracts showed similar and reproducible SDS-PAGE profile and resolved into 20 to 22 protein bands with molecular weights 10 to >245 kDa. However, E_{CF} extracts showed sharper protein bands as compared to E_{DT} extracts (Fig. 2). The proteins with molecular weight (M.W) 75 & 78 kDa showed higher band intensities in both E_{DT} and E_{CF} extracts. The proteins of M.W 88, 65, 48, 45, 36, 26, and 18 k Da were visible as prominent bands in both E_{DT} and E_{CF} extracts, similar to other reports for cockroach extracts (Thangnam *et al*., 2007; Tungtrongchitr *et al*., 2012). Present work indicates that the cockroach extracts dialyzed by centrifugal filtration are more consistent in terms of yield, protein & carbohydrate content, total recovery and showed a better and reproducible protein profile.

The SDS-PAGE is one of the effective electrophoretic techniques suitable for analysis of allergen extracts (Einarsson 1985). It not only assesses the identity of an allergen extract but may also provide valuable insight into changes or differences in components, structures or concentrations that cannot be deduced from other *in-vivo* or *in-vitro* biochemical analyses. Moreover, SDS-PAGE patterns are less susceptible to buffer or sample interferences and show reproducible alignments of protein components (Grier 2001). The commercial cockroach allergen extracts are reported to vary in protein content and electrophoretic banding patterns (Patterson *et al*., 2002).

The batch to batch reproducibility of cockroach extracts is essential for effective outcomes in allergic patient management i.e. diagnosis and immunotherapy (Tungtrongchitr *et al*., 2012). The biochemical and electrophoretic methods have the advantage of not requiring patient sera or immunologic reagents to obtain information pertaining to allergen content (Einarsson 1985). The SDS-PAGE and protein estimation are useful for identification and quality control of extracts while protein estimation is one of the important parameter to assess the potency also (Singh *et al*., 2001, Gaur *et al*., 2009). Modified Lowry's method is recommended for estimation of pollen and fungal extracts (Singh *et al*., 2001).

Conclusion

Minimal basic standardization procedures need to be implemented to regulate and check the inherent variations in cockroach allergen extracts obtained from naturally occurring biological source material in order to achieve uniformity and reproducibility in a clinical setting. The procedure(s) opted for extract preparation should be appropriately validated to resolve the batch to batch variability. The simple and easy to perform biochemical test parameters, i.e. protein and carbohydrate estimations, may be adopted to minimize the gross batch to batch variability in cockroach allergen extracts. Further, the reproducible SDS-PAGE pattern, as observed in present work may be a supportive and crucial quality control technique for identification and ensuring batch to batch consistency of commercial cockroach extracts. Similar approach may be considered for initiating quality control of other indigenous allergen extracts. However, further immunochemical quality control parameters need to be developed for optimal standardization of allergen extracts in India.

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