Generation of polyclonal antibodies against *Costus speciosus* (Koen ex Retz.) Smith phytoconstituents using peptide conjugated immunogen

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Abstract: This study has been undertaken to investigate the possibility of polyclonal antibody generation against *Costus speciosus* phytoconstituents. To test the anti-plant extract antibody production in rabbits, an innovative immunogen preparation was made for improving immune response to plant extracts. The immunogen preparation method involves incorporating albumin binding synthetic peptide polymer to phytoconstituents to enhance the systemic life of antigen and improve immune response against the phytoconstituents. The antibody generation in rabbit was evaluated by quantitative SDS PAGE and Western blot as well as by quantitative indirect ELISA. The results revealed the production of phytoconstituent specific antibodies which is an important tool for immunoassay development for detection of phytoconstituents in any *in vivo* study sample matrix. This is the first report for generation of antibodies to phytoconstituents from *Costus speciosus.*

Index Terms: *Costus speciosus*, plant extract, polyclonal antibodies

I. INTRODUCTION

Costus speciosus is a medicinal plant which is known to have anti diabetic property (Bavarva *et al.*, 2008). Plant extract constitute several plant product mixture and are called Phytoconstituents which comprises majorly of small molecule (<1000 Da) which cannot elicit immune responses. Most of the phytoconstituents are poorly immunogenic because they are low molecular weight compounds, a so-called hapten. In order to generate good quality antibodies, the phytoconstituents need to be made immunogenic by coupling with carrier macromolecules, such as proteins, or peptide polymers that binds to proteins which leads them to be recognized and be phagocytosed by antigen-presenting cells in the animal for production of polyclonal antibodies(Van Emon, 2017). It is important that the conjugated immunogen should preserve the common structure of phytoconstituents as much as possible to obtain high affinity specificity antibodies. Immunoassays are the important analytical tool in measuring quantity of antigen in any biological matrix; good quality antibodies play a critical role (Harlow and Lane, 1998). Recently, with the rapid development of the molecular biosciences and application in the field of Biotechnology, polyclonal antibodies are continuously of increased demand due to its nature of specificity and reactivity to mixture of antigen as in the phytoconstituents and hence used extensively in pharmacokinetics research, and quantitative and qualitative analysis of phytomedicine. The aim of this study was to evaluate and generation of antibodies to mixture of phytoconstituents using immunotechniques.

II. RESEARCH METHODOLOGY

Collection of *Costus speciosus* plant material

Whole plant from root to leaves of *Costus speciosus* were collected from Kinnikumri farm in the Western Ghats of Sullia Taluk, Dakshina Kannada district in Karnataka, India. A sample specimen is preserved at Vision Bioscience Private limited a Research and Development Laboratory in Bangalore. The collected leaves were cleaned, dried under the shade and powdered finely using grinder mill. Powder was sieved through particle size mesh of 40, stored at room temperature (26$\pm$2^{o}C) in cool dry place in an airtight polypropylene opaque bottle until use. The dried extract used for the current study.

Preparation of plant extract

Extraction of phytoconstituents from ~ 3 kilograms of air dried, sieved crude plant powder of *Costus specious* was performed using 90% ethanol by adopting marinating procedure for approximately 8 and half days in multiple flat bottom conical flasks with intermittent shaking on room temperature flask shaker. Extract was filtered and concentrated to dryness at room temperature to avoid the decomposition of natural metabolites. Dried extract were filled in Zip pack bags and preserved at 4^{o}C in a refrigerator (Samsung) till further use.

Preparation of immunogen

Phytoconstituents was accurately weighed and mixed w/w with albumin binding peptide polymers that was synthetically prepared and linked by EDC (1-ethyl-3-(3-dimethylaminopropyl carbodiimide) conjugation chemistry. Conjugated phytoconstituents, an immunogenic was prepared in 5g quantity for immunizing to rabbits for generation of polyclonal antibodies.

Generation of polyclonal antibodies

Polyclonal antibodies were generated in New Zealand white rabbits. Immunization scheme involved priming of animals with 100uL crude extract solution with complete Freunds adjuvant and boosted five times over a period of 2 months by subcutaneous injections of 100µl of the plant extract emulsified with equal volume of incomplete Freund's adjuvant (100µg of protein/ animal). One week after the last injection, animals were bled and sera was kept at<10°C until use.

SDS PAGE and Western blot

SDS PAGE (Sodium dodecyl sulphate -polyacrylamide gel electrophoresis). To understand SDS-PAGE profile of the extracted crude phytochemicals, an unit dimensional SDS-PAGE of 10% separating gel and 5% stacking gel was prepared and run in in a mini vertical system (Bio-Rad) . A Quantity of 10µg of solubilized crude sample was loaded in each sample well along with 5µl sample buffer containing bromophenol blue as tracking dye. A broad range marker was also run in the gel to determine the molecular weight of the bands. The gels were run at steady voltage of 100 V for 2.5 hours followed by silver staining (Silver stain solution from Affigenix Biosolutions, Bangalore). The gel was photographed and stored in 3% acetic acid.

For western blot, phytochemicals or proteins were transferred from polyacrylamide gel onto nitrocellulose membranes at 200 V and 150 mA in ice bath for 2 hours using Mini Protean II transfer cell (Bio-Rad, USA). Nitrocellulose membrane containing transferred phytochemicals were blocked with 10mMTris- buffer (TB) pH 7.2$\pm$ 0.05 and 3% Casein (Sigma) for 30 minutes. Washed membrane was incubated with mouse anti serum and rabbit antiserum separately (1:50 dilution) for 2 hours at 37ºC. Finally, washed membranes were incubated with respective anti-antibodies conjugated to Horse radish peroxidase (HRP) (Goat anti rabbit –HRP for rabbit antisera membrane; Rabbit anti mouse HRP for mouse antisera membrane; 1: 5000 dilution) for 1 hour at 37ºC. Enzymatic reaction was developed with TMB (Juniper, India) substrate. Image was captured subsequently in image scanner (GE healthcare Image master 2.0)

Antibody purification and quantification

Antibody purification from rabbit hyper immune serum was performed using Protein A/G column as per instruction manual (Thermo pierce, cat # 89954) independently and separately. Briefly, protein A/G column was loaded with anti sera /cell culture supernatant and incubated overnight in a tube rotator .Flow through was discarded and column was washed twice using Phosphate buffer saline pH 7.2. Bound antibody was eluted using 2M Glycine pH 2.5 and eluted antibody was collected in 0.1 M Tris pH 8.0. Collected antibody pools from rabbit sera were dialyzed in 3.5 kDa cutoff membranes (snake skin dialysis membrane, Thermo pierce; cat # 68035). Quantification of purified antibodies was performed by measuring the optical density of antibody solution in quartz cuvette at 280 nm using plate reader and spectrophotometer (M5E, Molecular devices with Spectramax software).

Antibody titer determination

Antibody titer was determined following an Indirect ELISA procedure where , the crude extract of plant was coated at concentrations in saline ranging from 1:2000 dilution , 2 fold to 1: 2048000 dilution , 100 µL / well onto high binding Maxisorp plates (NUNC) for 1 hour at room temperature. Washed plates were blocked using 3% PEG 8000 for 30 minutes. Anti serum of rabbit was diluted 1: 50 and added to separate plates with negative controls where the diluted anti sera was not added. Respective secondary antibodies to rabbit that are conjugated to HRP was added to plates and colored reaction after adding substrate was captured using plate reader at 450 nm. Obtained titer for rabbit antisera were reported.

III. RESULTS AND DISCUSSION

Phytoconstituents are organic chemical substances formed by plant cells. The separated/extracted chemical constituents have various applications in medicine and pharmacology which has made rapid stride and research has grown multi dimensionally and dynamically. So in the present study attempt has been made to evaluate various pharmacological, phytochemical and pharmacological parameters of the *Costus speciosus*. Similar studies with modifications were performed previously by academicians and researchers (Vishnu Bhat *et al.*, 2010). Ethanol extraction and dissolution of dried extract in sterile water for further studies were previously reported by Eliza *et al*., (2009).

Generation of anti phytoconstituents polyclonal antibody pool by conjugating the extracted phytoconstituents from *Costus speciosus* plant with albumin binding short peptide. When this conjugate is injected to animals for antibody production, the albumin binding site binds to circulating albumin in the animal system increasing the stability of the conjugated phytoconstituents pool and presenting the paratroopers of antigen pool for antibody generation efficiently without losing the antigenic property unlike when unconjugated phytochemical pool is immunized for antibody production. This helps in generating antibodies for majority of the antigens in the pool of phytoconstituents. Antibodies for phytoconstituents were developed in New Zealand rabbits at Liveon Bioloabs laboratory for preclinical studies, Tumkur, India.

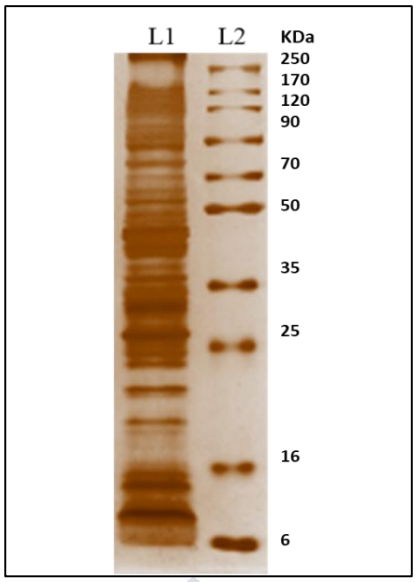

Figure 1: Silver stained profile of extracted phytoconstituents

SDS PAGE of 12% with broad range molecular weight marker was run to evaluate the profile of proteins, amino acids and other separable molecules in the plant extract. Resolved phytoconstituents were silver stained as per standard procedures and image was captured. A wide molecular weight range phytoconstituents were observed on silver stained gel (Figure 2).

Western blot was performed to evaluate the reactivity of anti serum to the phytoconstituents which enables development of immunoassays to detect the plant extracts in circulation when plant extract is used as treatment. A considerable number of bands appeared due to specific reactivity of antibodies in antisera and the phytochemicals resolved and transferred on the nitrocellulose membrane for blotting. The western blot profile obtained were developed using substrate and the image was immediately captured (Figure 3).

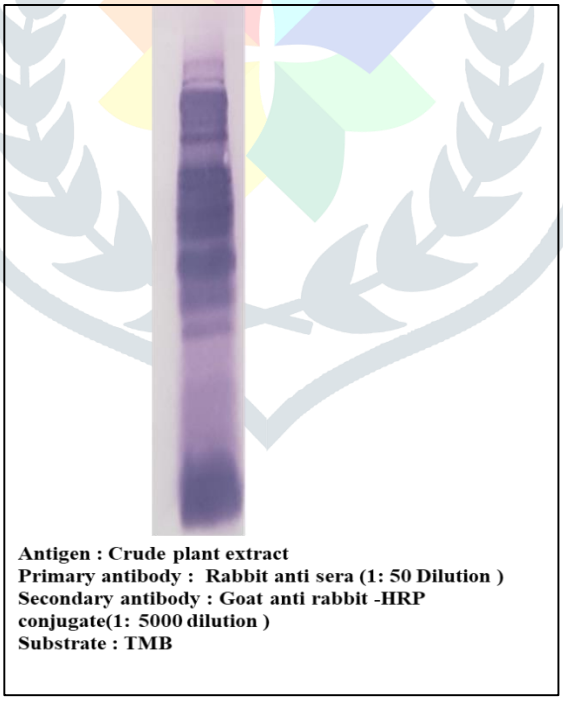

Figure 2: Western blot of phytoconstituents

SDS PAGE and a Western blot performed for extracted phytoconstituents revealed a proteome profile ranging from 25 KDa to 6 kDa. Western blot using rabbit serum had > 60 % coverage of binding to specific separated phytoconstituents. Specificity by western blot was found promising for development of Immunoassays to detect circulating plant extract in serum/plasma after treatment and also indicates the possibility of anti extract antibodies to be generated during long term preclinical study

Titer of the antibody in the antiserum was evaluated by Indirect ELISA procedure. Titer is the reciprocal of the highest dilution of the antibody/antiserum that gives response equivalent to the negative control sample. A serial dilution of

antiserum starting from 1:2000 dilutions in two fold ten serial dilutions were performed and analyzed. Figure 3 graphically represents serially diluted sample results. Titer of rabbit anti serum was found to be 1/1024000.

Generations of antibodies to plant products are rare due to its complexity in molecular and biochemical composition leading to antigenicity. However, unique immunogen preparation has led to successful development of polyclonal antibodies to phytoconstituents. This is the first report of generating antibodies against Costus speciosus plant products and evaluating antibodies to the phytoconstituents.

Antibody generation studies are very rare across the globe for plant products as a whole. Further the antigenic characterizations by proteomic profiles in PAGE, western blot and indirect ELISA for anti sera reactivity and titer assessed in this study indicate antibody suitability for Immunoassay development for bio analytical application.

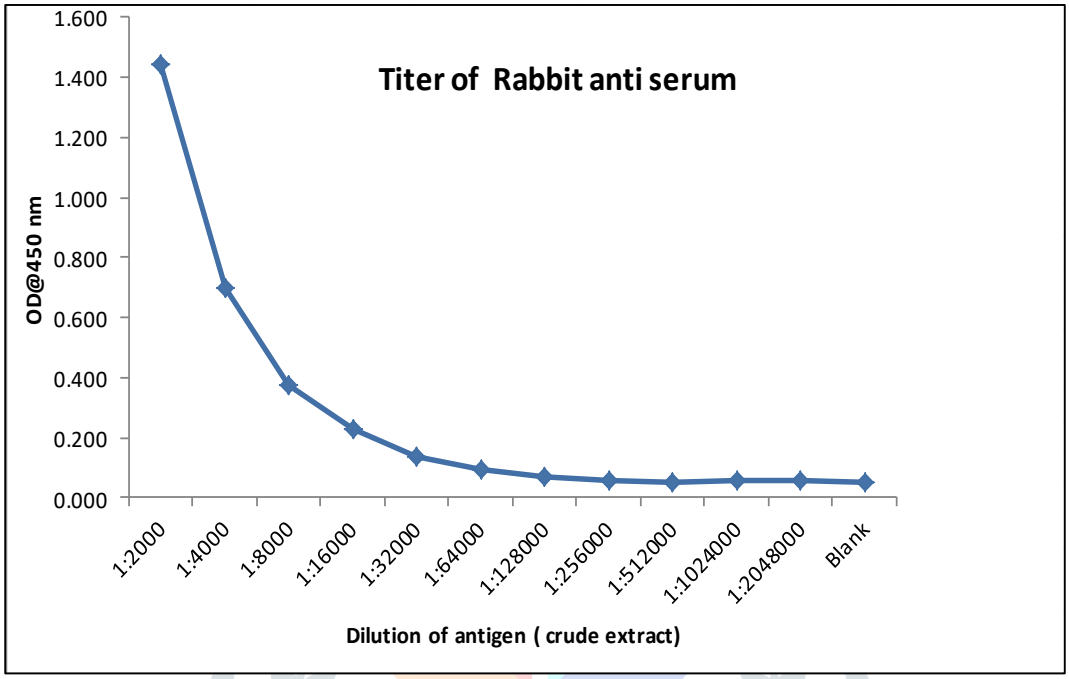

Figure 3: Titer of rabbit antiserum

This study provides a strong basis to evaluate Immunoassay methods, such as ELISA, immunoaffinity chromatography, and chromatographic immunostaining as an important analytical tool for phytoconstituents characterization.

IV. CONCLUSION

This is the first report of generating antibodies to Costus speciosus phytoconstituents. This study is also a first report of using albumin binding peptide polymers to conjugate phytoconstituents in enhancing immune response for antibody production. The outcome of the study indicates that the antibodies generated are useful tool in techniques involving immunoglobulin mainly immunochemistry and bio analytical assessment of mixture of phytoconstituents in study matrix.

V. ACKNOWLEDGMENT

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VI. REFERENCES

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