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Original Article

LEGUME INDIAN BEAN (*PHASEOLUS VULGARIS*) PARTIALLY PURIFIED ANTI-NUTRITIONAL PROTEIN FACTOR EXHIBIT ANTI-OXIDANT, IMMUNOMODULATORY AND ANTI-CANCER PROPERTIES

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ABSTRACT

Objective: This study was aimed to access one of the hidden ANF in Indian bean and assessment of its antioxidant, immunomodulatory, antiangiogenic, and anti-cancer properties.

Methods: Indian beans were processed and evaluated for the presence of ANF through HA activity. Bean-ANF was partially purified by ammonium sulfate precipitation (ASP) and dialysis. Obtained Partially Purified bean-ANF (PPb-ANF) resolved on 12% SDS-PAGE, analyzed protease digestibility. The immunogenicity of PPb-ANF was characterized by producing polyclonal antibodies and checked for the presence of natural antibodies in healthy human sera. Pharmacological studies of PPb-ANF were assessed through anti-oxidant, immunogenic, anti-angiogenic and anti-cancer assays.

Results: Partially purified Bean-ANF (PPb-ANF) showed significant specific HA activity up to 150 Units/mg compared to crude extract 9.3 of Units/mg. The PPb-ANF was identified to have more than 90% homogeneity in purity with an observed M W of 30-32 kDa. PPb-ANF showed significant pepsin digestive stability up to 2 h at 37 ºC and appears digestive resistant. Rabbit produced anti-PPb-ANF IgG polyclonal antibody on immunization and exhibited immunogenic properties. Natural human IgG and IgE antibodies were found in the non-atopic human sera against PPb-ANF, indicative of its immunogenicity. Pharmacological studies of PPb-ANF confirmed its potent antioxidant activity. At 400 µg of PPb-ANF showed the highest around 90% DPPH activity and at 150 µg of PPb-ANF showed 89% inhibitory lipid peroxidation activity. The PPb-ANF showed 70% (3 µg) and 40% (1.5 µg) inhibitory in blood vessel density compared to control and exhibited significant anti-angiogenic activity. The PPb-ANF showed a significant (*p<0.05.) reduction in solid tumor weight upon dose-2 treatment (50 mg/kg body weight) and also increased lifespan up to 70 d compared with the control tumor model.

Conclusion: PPb-ANF was identified as digestive-resistant, hemagglutinin with a molecular weight of 30-32 kDa. The protein is immunogenic and has potential antioxidant, antiangiogenic and anti-cancer properties. Further purification and mechanistic-based pharmacological characterization might explore this protein as a potential anti-cancer molecule.

Keywords: Anti-nutritional factor, Antioxidant, Anti-angiogenic, Anti-cancer, Immunomodulatory

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INTRODUCTION

Legumes deliver the highest source of dietary protein in many parts of the globe. Legume grains incorporate 17-40% of total protein contents, which is co-equal to 18-25% of meat protein contents. Unfortunately, the existence of anti-nutritional factors (ANFs) in legumes truncates the exercise of feasible proteins and carbohydrates [1]. These ANFs are structurally non-identical compounds having low-grade protein quality allied with a declensional part of sulfur amino acids and also inbuilt with down-level inherent protein digestibility [2]. Anti-nutritional factors are compounds that limit the exploitation of nutrients in plant food products for humans and animals. They play a responsible role in determining the nutritional value of plants. Plants incorporate these ANF substances to safeguard and meanwhile to eradicate themselves from being eaten. If diet patterns contain some of these ANFs, they amplify the harmful effects on the human body. To maintain the nutritional values of food products, these ANFs are required to be deactivated or eliminated. Plants which produce seeds called legumes are luxurious in energy-producing carriers, namely proteins; carbohydrates and lipids to protect these energy carriers; plants naturally build up with active chemical defense substances, nothing but ANFs. Legume grains also contain significant amounts of ANFs such as lectins, non-protein amino acids, protease inhibitors, saponins, alkaloids, pyrimidine glycosides, cyanogenic glycosides, isoflavones, tannins, oligosaccharides, phytates [3]. Along with these ANFs, poisonous substances like cyanides, phenols, and nitrates are also present in several plants. Cassava is one of the plants that contain high amounts of cyanides, it is a poisonous respiratory compound [4]. Intake of these plant food products in their fresh form is accepted to contain the highest micronutrients than in their processed form; this has turned ought to be a major health complication because of the occurrence of high profile-ANFs and toxic substances [5]. ANFs can be portioned into two groups; namely heat-stable ANFs contains tannins, phytic acids, alkaloids, non-protein amino acids, saponins, etc., and heat-labile ANFs contain lectins, protease inhibitors, cyanogen glycosides, and toxic amino acids, etc. [6]. Concentration of attention mainly on legume Anti-nutritional factor; these are mostly carbohydrate-binding proteins which are exclusively recognized and explore diverse sugar structures and meanwhile umpire a line-up of biological processes including cell-cell interaction, host-pathogen interaction, serum-glycoprotein output and finally deals with innate immune responses [7]. Furthermore normal cell growth, development, and differentiation processes connect many types of cellular interactions among and between cells in insoluble or soluble macromolecules. These kind of interactions were regulated by specific cell-surface components, some of which contain carbohydrates as their cell-

surface components. This legume ANF is a divalent or polyvalent carbohydrate-binding proteins in nature that are able to bind as well as precipitate and agglutinate glycoproteins [8]. *Phaseolus vulgaris* belonged Indian beans and were employed generally in the daily diet of humans as well as animals across the world, also recognized as cheap and best leading protein source with high fiber and low fat contents [9]. But due to the presence of anti-nutritional factors in beans extra care should be taken while cooking and processing in order to felicitate the beans to fit for human consumption [10]. These ANFs in legumes are good enough resistant to digestive enzymes and a limited extent, resistant to thermal denaturation. As mentioned earlier these anti-nutritional epic family legumes having carbohydrate-binding proteins. Apart from their anti-nutritional property, they are also capable of exhibiting copious biological activities such as anti-fungal, anti-angiogenic, anti-tumor, and anti-HIV activities [11, 12]. A series of research surveys on ANF have been conducted to reveal their blood grouping capability, erythro and leucocyte agglutination activities, stimulation of mitogenic-lymphocytes, cells, and other particles-fractionation, lymphocyte subtype studies, normal-pathological histochemical condition studies. Further studies also conducted for the induction of IgE production in rats through intragastric administration [13]. It is yet to be discovered whether the existence of ANF in legume beans is a boon or a curse is still in research progress. Overall, beneficial and non-beneficial characteristics of ANFs in legume beans are still to be explored. The present study aimed to investigate and explore both beneficial and non-beneficial inbuilt activities of ANF in legume beans like Erythro and Leuco-agglutination capabilities, anti-oxidant properties, immunogenic ability, allergenic potency, anti-angiogenic properties and finally with anti-cancer potentialities.

MATERIALS AND METHODS

Materials

Ultracel-3 regenerated cellulose membrane Amicon centrifugal tubes were purchased from HiMedia laboratories, in Mumbai, India. Freund's complete-adjuvant and Freund's incomplete-adjuvant, ALP-Tagged goat anti-rabbit IgG monoclonal antibodies, ALP-labelled mouse anti-human IgE monoclonal antibodies, ALP-conjugated mouse anti-human IgG monoclonal antibodies were obtained from Sigma-Aldrich, St. Louis, MO, USA. The Nitrocellulose membrane was procured from Amersham Protran, Merck, Bangalore, India. MICROLON flat-bottomed ninety-six-welled microtiter plate was obtained from Eppendorf. Protein-A agarose was obtained from Bangalore Genei, Bangalore India. Remaining all other experimental reagents and chemicals used in this research study were of analytical grade and were purchased from Sigma-Aldrich, HiMedia, and SRL, India.

Purification and characterization of bean anti-nutritional factors (ANFs)

Collection and preparation of bean saline extract

Phaseolus vulgaris belonged dry-Indian legume navy bean seeds that were procured from the Shivamogga local market. The collected bean samples were authenticated by the botanist and the sample was submitted to the depository at Sahyadri Science College, Kuvempu University, Shivamogga, India. Voucher specimen number of dry navy bean seeds (Voucher no. KU/SSC/BOT/210/1/2017-18) 100 g of bean seeds were dried for two days in sunlight to avoid moisture and finely homogenized to powder form by using a mixer grinder at full speed for 5 min and Sieved for better yield. 50% of w/v bean-saline extract was prepared by weighing 50 g of bean powder in 500 ml of 10 mmol Phosphate buffer saline with pH 7.4 in a 1000 ml beaker with a magnetic stir bar and suspension was stirred for about 16-24 h at 4 ºC. Thoroughly dissolved bean saline solution was filtered by using 2-3 layers of muslin cloth and collected filtrate was centrifuged at 10000 rpm at 4 ºC. a clear solution with slight buffy-brown colored supernatant was collected and stored at 4 ºC, it was referred as crude bean saline extract.

Ammonium sulphate fractionization and dialysis

The obtained clear bean-saline extract was further purified by subjecting to sequential 0-20%; 50-90% ammonium sulphate precipitation as per the previously followed methods with slight modifications [14]. Briefly, a measured saturation weight of ammonium sulphate crystals were added to bean-saline supernatant for 20% saturation with gentle agitation using a magnetic stirrer at 4 ºC for 60 min and undisturbed overnight at 4 ºC. Completely dissolved ammonium sulphate buffer bean saline solution was centrifuged at 3000 rpm for 20 min and the precipitate was collected. Obtained 20% precipitate was resuspended in 2-3 ml of 10 mmol PBS with pH 7.4: further continued for the addition of ammonium sulphate to obtain 50% and 90% saturation at 4 ºC. The obtained precipitate mixture was dialyzed against 10 mmol PBS to recover the concentrate using an Amicon stirred-cell Ultrafiltration unit with 3 K molecular weight cut-off (MWCO) to drain off low-molecular-weight compounds and salts. The purified fraction was labelled as a Partially purified bean-Anti nutritional factor (PPb-ANF).

Erythro-agglutinating and Leuco-agglutinating capacity of crude and PPb-ANF

Preparation of 1%-Trypsinized RBC suspension

5-10 ml of Fresh chicken blood was sampled from a local chicken slaughterhouse and 2-5 ml of rabbit blood sample was also drawn. Both blood samples were collected in separate sterile test tubes containing an equal volume of Alsever's solution. Further, the test tubes were centrifuged at 1000 X g for 10 min at 4 ºC and collected pellet, discarded the supernatant. Pelleted erythrocytes were washed with 0.9% NaCl saline 3-4 times and resuspended finally in 10 mmol PBS with pH 7.4. 1% Trypsin was added to RBC suspension and incubated for 10 min at 37 ºC and trypsinized cells were again centrifuged for 10 min at 1000 X g at 4 ºC and subjected to saline wash. Prepared 1% trypsin-treated erythrocyte suspension was further used for HA assay [14].

Erythro-agglutinating activity

The hemagglutination activity of PPb-ANF was examined using 1% trypsinized chicken erythrocytes. In brief, 50-100 µl of 1% suspension of trypsin-treated chicken erythrocytes were taken in an agglutination plate containing 5-10 µg of PPb-ANF in 10 mmol PBS with pH 7.4 Mixed gently and incubated for 60 min at 37 ºC, visualized the agglutination of RBC-suspension. Erythrocytes only with buffer and without the addition of protein extract were used as control. The minimum quantity of PPb-ANF necessary to agglutinate RBCs (one HA unit) and Total HA activity was also analysed shown in table 1.

Leuco-agglutinating activity

The leuco-agglutination activity of PPb-ANF was carried out with the help of human peripheral blood lymphocytes. Briefly, 2.5 ml of Hisep-Lymphocyte separation medium was taken in an aseptic 15 ml centrifuge tube and carefully added 7.5 ml of diluted chicken blood and was then subjected to centrifugation for 30 min at 400 x g. carefully aspirated separated lymphocyte layer by the addition of equal volume of 10 mmol Phosphate buffer saline with pH 7.4 and again subjected to centrifugation for 10 min at 260 x g. 200 µl of separated lymphocytes in 10 mmol Phosphate buffer saline were taken into the clean concavity agglutination plate and added 0-20 µg of PPb-ANF, mixed gently further continued the incubation at 37 ºC for one h. Agglutinated leucocytes were visualized under optical light microscopy and photographed.

Protein quantification and SDS-PAGE protein profile of PPb-ANF

Protein estimation was done by Standard Lowry's method of protein assay using 1 mg/ml Bovine serum albumin stock solution prepared using distilled water and diluted working solution was taken as a control rising from 40 µg to 200 µg concentration. 5-10 µg of the above-mentioned test samples were taken into the test tubes and uniformed with distilled water. Further continued by the addition of alkaline copper reagent (2% sodium carbonate prepared in 0.1 N NaOH, 0.5% copper sulfate, 0.5% sodium potassium tartrate) for 30 min incubation at room temperature in a dark place. Finally, 1:1 diluted Folin–Ciocalteu reagent (FC-reagent) was added with distilled water and the absorbance was read at 660 nm in a spectrophotometer. Concentration or percent values were

mentioned in table 1. 5-10 µg of crude, ammonium sulfate fractioned and dialyzed PPb-ANF was subjected to 12% reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis using Bio-Rad mini electrophoresis unit. Staining was done by using Coomassie brilliant blue R-250.

Pepsin digestibility of PPb-ANF

The digestive stability of PPb-ANF was determined by using pepsin (2500 units activity/mg) by following the previously described methodology [15]. Stimulated gastric fluid was prepared with 0.084 N HCl and 35 mmol NaCl and pH was adjusted to 1.2 with dilute HCl and NaOH along with 2500 pepsin activity units per mg test proteins. The pepsin digest is performed by mixing 1.9 ml of SGF containing 2500 pepsin activity units with 0.1 ml of PPb-ANF (5 mg/ml) and mixing well before placing it on 37 ºC. A control sample was taken without the protein and protein mixed with SGF without pepsin was also performed to evaluate auto-digestion. During the incubation time at regular intervals of 0, 5, 10, 20, 40, and 60 min, 200 µl of aliquots of the digest were taken out into a tube containing 70 µl of NaHCO3 buffer pH 11. 6 X Sample dye was added to the samples obtained, mixed well and heated for 5 min. Zero min digest is the solution of pepsin quenched before adding the test protein. All the aliquots were analyzed by SDS-PAGE 12% gel under reducing conditions. The results were observed and documented.

Free-radical scavenging potentiality of PPb-ANF

Anti-oxidant activity of PPb-ANF extract was analyzed by using the DPPH method assay following, as mentioned previously [16]. with slight modifications. Briefly, 24 mg of DPPH stock solution was prepared using 100 ml of 95% methanol and a working solution with absorbance 0.973 at 517 nm was prepared by diluting with methanol. 12.5, 25, 50, 100, 200, 400 µg concentrations of DPPH working solution were taken in 6 test tubes and ascorbic acid was used as a standard for better comparison. Known concentration (5, 10, 15, 20, 25 and 30 µg) of PPb-ANF was added in another set of 6 test tubes (unknown) and incubated for 30 min in a dark place. After 30 min of reaction, all the samples were read at 517 nm in a spectrophotometer. The percentage of radical scavenging activity was determined by using the formula. Percentage of antioxidant activity $= \{A_C - A_T\} \div (A_C) \times 100$

Where A_C–is the absorbance of the control, A_T–is the absorbance of the test sample.

Lipid peroxidation inhibition percentage of PPb-ANF

Inhibition of Lipid peroxidation chain reaction by PPb-ANF was investigated using the TBARS method as previously described with slight modification [4]. In brief, 8-10 w-old healthy Swiss albino mice with 26-30 g weight were sacrificed by subjecting to over-ether anesthesia humanely, dissecting the liver, and weighing. From that 10% of liver, homogenate was prepared with 0.15 M KCl. 0.5 ml of 10% liver homogenate and 2 ml of PPb-ANF extract with 25, 50,100, and 150 µg concentrations were taken in test tubes. Added 100 µl of 0.2 mmol ferric chloride to all test tubes and incubated for 30 min at room temperature to induce peroxidation reaction in homogenated lipid. The reaction was stopped by the addition of 2 ml of ice-cold 0.25 N HCl containing 15% TCA, 0.5% BHA, 0.38% TBA, and mixed thoroughly, then subjected to boiling water for 60 min. Cooled the reaction mixture and centrifuged at 3000 rpm for 10 min. Absorbance of each supernatant was measured at 532 nm and calculated the inhibition percentage of lipid peroxidation by using a formula. Percentage of Inhibition $= \{A_{control} - A_{test}\} \div (A_{control}) \times 100$.

Where, $A_{control}$–is the absorbance of the control tube, A_{test}–is the absorbance of test tube.

Animal-based experiments

All animals (New Zealand white rabbit and Swiss-albino mouse were procured from Live On Bio Labs-Laboratory animal supply, Antharasanahalli, Tumkur-572106; animals were maintained in special humidified animal house for acclimation). Experimentations incorporated in the present research study were approved by Institutional Animal Ethical Committee (IAEC), According to the committee purpose of CPCSEA guidelines for laboratory animal facilities. (Ref. No. KSHEMA/IAEC/01/2020 Date: 04.06.2020).

Production of polyclonal antibodies against PPb-ANF

Generation and accumulation of polyclonal antisera to PPb-ANF in rabbits polyclonal antibodies against 1:1 ratio emulsified PPb-ANF was hoisted in rabbits by injecting 0.5 ml of 100 µg protein concentration of PPb-ANF immersed in an equal quantity of Freund's complete adjuvant with potent immune enhancer mycobacterial cell wall subcutaneously. After 15 d duration of primary injection, Freund's incomplete adjuvant without enhancer was given continuously as booster doses (3 booster doses) intramuscularly at regular 7 d intervals of time with 1:1 ratio emulsified PPb-ANFF (50 µg) with incomplete adjuvant. For every dose of immunization, collected blood samples from rabbit by puncturing the marginal ear vein and obtained immunized blood and also collected pre-immunized blood, which was used as control. Blood was kept at 4 ºC without any disturbance for 12-24 h and centrifuged at 10,000 rpm and; collected the pale yellow with a slightly red colored serum layer was aliquoted the pooled serum and maintained at-20 ºC storage.

Partial purification of anti-PPb-ANF serum polyclonal-IgG antibody and protein quantification

3 ml of Immunized serum was subjected to 0-80% Ammonium sulphate precipitation with a corresponding saturation amount of ammonium sulphate and dissolved completely, further incubated overnight at 4 ºC. incubated sample was centrifuged at 5000 rpm for 20 min maintaining 4 ºC. collected pellet was resuspended in 10 mmol phosphate buffer saline with pH 7.4. The resuspended pellet was further purified by subjecting it to dialysis. In brief, the total pellet suspension was transferred to an activated dialysis membrane (3 K MWCO) and checked for no leakage. The dialysis membrane was fitted to a glass rod and placed in 1000 ml of saline prepared with 9 g of sodium chloride. Fixed speed of circular motion was set up by magnetic stirrer and changed saline solution for every one hour. Finally collected end product was after 24 h of dialysis. Protein quantification of crude, ammonium sulphate precipitated and dialyzed rabbit immunized serum was done by using Lowry's method with BSA as standard.

Immuno-affinity chromatography purification and SDS-PAGE

Dialyzed Immunized serum was further purified by using Staphylococcus protein-A agarose polymer in affinity column chromatography to isolate serum polyclonal IgG antibody. Initially, the agarose bed was equilibrated with a 1:10 ratio bed volume using equilibrium buffer 0.05 M PBS pH 7.4. An equal volume of dialyzed immune serum was mixed with 1 X equilibrium buffer and centrifuged at 10,000 rpm and equilibrated clear serum was loaded onto the protein-A agarose column 0.2 ml/min. Repeated the wash-through fraction cycles 2-3 times and unbound serum IgG were collected at the flow rate of 1 ml/4 min by passing twenty-five-bed volumes of 1 X equilibrium buffer. 1:5 bed volume of 1 X 0.05 M citrate elution buffer, pH 3 was passed through the column at the flow rate of 1 ml/4 min and the eluted fractions were collected in 1.5 M Tris, pH 10 neutralization buffer. Affinity-purified serum IgG was dialyzed against PBS, pH 7.4 and stored with 0.1% sodium azide at-20 ºC.

Purified anti-PPb-ANF antibody profile by SDS-PAGE

Every collected fraction in the affinity column was checked for total IgG concentration at an absorbance of 280 nm by Lowry's method of protein quantification using BSA as standard. Isolated anti-PPb-ANF IgG antibody homogeneity was checked by subjecting 12% SDS-PAGE under reducing conditions, using Bio-Rad mini electrophoresis unit and stained by coomassie-brilliant blue R-250.

Ouchterlony double-immunodiffusion assay against PPb-ANF antigen

A suitable glass container (25 mm Petri dish) for agarose gel diffusion studies was prepared by using 1.2% agarose dissolved in 10 mmol PBS of pH 7.4 and allowed to boil at 100 ºC and added

preservative 1% sodium azide. Warm agarose solution was poured onto a 25 mm Petri dish and kept undisturbed for 30 min to form an agarose gel bed. 5 Wells were punctured at a minimum of 1 cm distance apart from another well with approximately 100 µl well capacity by using round shaped hollow steel bar and templates were carefully withdrawn with forceps. For immunodiffusion assay, 10 µg concentration of antigen PPb-ANF (crude, ASP 0-20%, ASP 50-90%, PPb-ANF) was loaded onto four different wells. In the middle of the four wells immunized serum was added. Antigen-antibody in the agarose gel was kept undisturbed for 24 h for immunodiffusion in a humidified 37 ºC environment and kept under observation continuously. Opaque precipitation bands started to appear between 12-24 h and were clearly visible.

Dot-immunoblot analysis

The expression of PPb-ANF was evaluated using a standard dot-immunoblot assay. In brief, the first set with 10 µg of PPb-ANF (1 µg/µl) and negative control BSA. The second set with 10 µg of crude, ASP 0-20%, ASP 50-90%, and PPb-ANF were spotted on the Nitrocellulose membrane with 0.45 µm pore size (Bio-Rad Laboratories) and favored to air dry. Un-blocked active target binding sites on the membrane were blocked by incubating for 1 h with 5% Skimmed milk powder in 10 X TBST buffer (10 mmol Tris-Hcl pH 7.5, 0.1% (v/v) Tween-20, 145.4 mmol NaCl). Blocked membrane after 1 h was washed with diluted 10 X PBST buffer (0.2 M potassium phosphate, 1.5 M sodium chloride, 0.5% (v/v) Tween-20, PH 7.2) and incubated for 12 h or overnight at 4 ºC with diluted rabbit immunized serum in 5% skimmed milk powder with TBST. Again given a wash and continued by incubating the membrane with ALP-conjugated anti-rabbit-IgG tagged antibody produced in goat at 1:5000 dilution for 1 h at 37 ºC. The membrane was washed with PBST and incubated with a combination BCIP (Nitro blue tetrazolium)/NBT (5 Bromo-4 chloro-3 indolyl phosphate) substrate kit. Furthermore, pre-immunized serum was taken as negative control.

Western blot analysis

10 µg of Crude extract and PPb-ANF was subjected to 12% SDS-PAGE under reducing conditions using Bio-Rad mini electrophoresis unit for 1 h. Resolved Proteins on SDS-PAGE were transferred onto a nitrocellulose membrane using a western blotting unit for 1 h. Protein transfer will be confirmed by ponceau staining. The membrane was collected carefully and blocked using 5% skimmed milk powder in 10 X TBST buffer, continued incubation for 1 h and washed with 10 X TBST buffer (10 mmol Tris-Hcl pH 7.5, 0.1% (v/v) Tween-20, 145.4 mmol NaCl). Further, the membrane was incubated for overnight with 1:10 diluted rabbit sera at 4 ºC. After washing, the membrane was incubated with ALP-Tagged goat anti-rabbit IgG monoclonal antibodies followed by the addition of 100 µl of BCIP (Nitro blue tetrazolium)/NBT (5 Bromo-4 chloro-3 indolyl phosphate) substrate and observed the band visually.

Interaction of PPb-ANF with natural rabbit IgG-antibodies and Human IgG, IgE-antibodies

Antigenic Titre of PPb-ANF against rabbit serum

Polyclonal antisera against PPb-ANF antigenicity was checked by using an In-direct ELISA assay. In brief, 96 microtitre ELISA plate was coated with serially diluted PPb-ANF (5 µg to 5 mg) in 100 µl of 0.05 M carbonate-bicarbonate buffer, pH-9.6, after 16-24 h incubation at 4 ºC microtitre plates were washed with PBS-T (0.05 M PBS, pH 7.4, 0.1% v/v Tween-20) followed by the addition of blocking buffer 1% BSA in PBS-T and incubated for 1 h at 37 ºC. Again the plates were washed with PBS-T and added 100 µl of 1:100 diluted rabbit serum with 1% BSA in 0.05 M PBS-T (0.1% v/v) to each well, incubated 12-24 h at 37 ºC. rabbit serum in ELISA microtitre plate was washed completely with PBST 3 times and added 100 µl of ALP-labelled goat anti-rabbit IgG monoclonal antibody (1:2000 dilution) to each well, incubated for 1-2 h at room temperature. Finally was added with 100 µl of 1 mg/ml PNPP substrate and the further reaction was stopped by adding 50 µl of 3 N NaOH; absorbance was read at 405 nm using a microplate reader and recorded.

Collection of healthy human serum samples

4-6 ml of healthy human blood sampling were drawn by clinically expert technicians, including all blood groups in-between the age of 25-32 with respective seven volunteers and also obtained consent form by them before sampling the blood. Selected individuals disclosed none of the case histories were recorded on legume allergy. Withdrawn blood samples were kept at 4 ºC for 16-24 h and centrifuged at 1000 rpm for 10 min at 4 ºC. clear yellow serum on the top layer of the RBC pellet was collected carefully and stored at-20 ºC. Serum samples were further used for IgE/IgG specificity.

Healthy human serum IgG-antibody interaction with PPb-ANF

An In-direct ELISA assay was performed to screen the specificity of IgG-antibody against PPb-ANF. Briefly, 10 µg of PPb-ANF in 100 µl of 0.05 M sodium bicarbonate buffer with pH 9.6 were coated onto the microtitre ELISA plate and incubated for 12-24 h at 4 ºC. washed the plate with 0.05 M PBS containing 0.1% w/v Tween-20 and subjected to blocking with the addition of 5% skimmed milk powder or 1% BSA to the same buffer for 1 h. Washed the plates, followed by the addition of 100 µl of 1:20 diluted human serum samples named S1-S7 and incubated overnight by maintaining 4 ºC. after the PBST wash added 100-200 µl of ALP-labelled monoclonal Anti-human IgG-antibody (1:30,000) dilution and again incubated for 12-24 h at 4 ºC, finally 100 µl of PNPP substrate (1 mg/ml) was added and 3 N NaOH solution used as stop solution. Measured absorbance at 405 nm and plotted the graph.

Healthy human serum IgE-antibody interaction with PPb-ANF

Performed Indirect ELISA to screen the specificity of IgE-antibody against PPb-ANF in 7 healthy individual serums. In brief, the microtiter ELISA plate was coated with 5-10 µg PPb-ANF in 100 µl of 0.05 M carbonate-bicarbonate buffer with pH 9.6 and incubated for 12-24 h at 4 ºC. washed microtiter plate with PBST (0.05 M PBS containing 0.1% w/v Tween-20 with pH 7.4) and blocked with the addition of 5% skimmed milk powder or 1% BSA for 1 h. Again, washed and added 100 µl of human serum samples S1-S7 (1:20 diluted) followed by incubation at 4 ºC for 12-24 h. After PBST wash, 200 µl of ALP-tagged monoclonal Anti-human IgG antibody (1:30,000) was added and further incubated overnight at 4 ºC. Added finally 100 µl of PNPP substrate, 3 N NaOH stop solution and read the absorbance at 405 nm and plotted graph.

Anti-angiogenic property of PPb-ANF

PPb-ANF angio-prevention potentiality was analyzed by using the In-Ovo CAM assay. Briefly, fertilized zero-day chicken eggs were obtained from the veterinary college, shivamogga and incubated in a humidified incubator chamber at 37 ºC. angiogenic fertilized eggs were cut-opened on 4th day of incubation with a sterile scalpel into a small square-shaped window on top and were treated with filter disc containing 1.5 µg of PPb-ANF as 1 dose, 3 µg as 2 doses and control without PPb-ANF. The cut-opened square-shaped window layer was covered with a sterilized coverslip and re-incubated for 72 h at 37 ºC in humidified condition. Vascularization changes occurred in incubated control and PPb-ANF-treated eggs were observed and photographed.

Anti-cancer activities of PPb-ANF

Cancer-regulation capacity of PPb-ANF was performed in solid tumor model animals by following methods as mentioned previously [17]. Briefly, Ehrlich ascites carcinoma (EAC) cells were procured from National Centre for Cell Sciences (NCCS), Pune (India). EAC cells ($1x10^6$ cells/Mouse) were grown and developed in Balb/c male mice weighing up to 27-30 g by injecting intra-muscularly to the right thigh portion of the hind limb. Subsequent observable enlargement of tumor size in the hind limb of administered animals was picked spontaneously for control as well as test animals: control, dose 1, dose 2 (n =6/group). Regular six doses on alternative days of tumor suspension lectin PPb-ANF with dose 1(25 mg/kg body weight), dose 2 (50 mg/kg body weight) was intraperitoneally administered with a 26 gauge needle and tumor growth was analyzed continuously with the help of a vernier caliper. Survivability rate, tumor volume, and weight differences were determined by comparing with control and test models.

Statistical analysis

The data were analyzed and graphs were made using MS Excel version 10. Statistical significance was evaluated by one-way

analysis of variance (ANOVA) followed by Student's t-test. Values are expressed as a mean of triplicate values±standard deviation (SD). Values of *P<0.05 were considered statistically significant.

RESULTS

Purification and characterization of PPb-ANF

Crude bean saline extract was obtained and checked hemagglutination capacity. Initially, bean extract activity was confirmed in chicken erythrocytes, where the results interpreted that bean saline extract immensely agglutinated chicken erythrocytes (fig. 1C). Bean saline extract protein quantification confirms the existence of enormous proteins (5290 mg) and was further purified by subjecting to 0-20%, 20-50%, and 50-90% ammonium sulfate fractionization. All fractions obtained in ammonium sulfate precipitation were checked for the presence of bean-ANF by subjecting them to hemagglutination activity. Among them 50-90% fraction showed the greatest agglutination capacity and confirms the existence of ANF. 50-90% fractionization yielded 55.8% of total bean proteins and were in purified form (fig. 1A and B). These proteins were further purified by subjecting them to dialysis using a 3 K cut-off Amicon ultracentrifugation unit to obtain the concentrated bean proteins. Overall protein concentration, specific activity, Total Hemagglutination activity, and recovery percentage in each step protein of purification were mentioned in the table 1. Bean extracts protein band separation based on molecular weight was checked successfully by SDS-PAGE, which were almost 90% of homogeneity with a slight contamination of high molecular weight proteins under reducing condition. SDS-PAGE molecular mass estimation of PPb-ANF revealed a band at 30-32 kDa molecular weight (fig. 1B). Lectin activity of PPb-ANF was checked in both erythrocytes as well as lymphocytes. PPb-ANF agglutinated RBCs and lymphocytes greatly at the maximum levels and the interaction of lymphocytes with PPb-ANF was visualized under a microscope (fig. 1C, D and E).

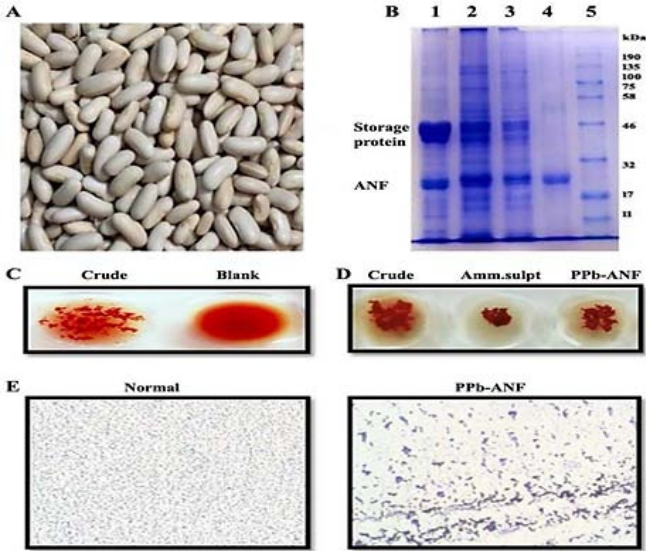

Fig. 1: Partially purified bean-ANF protein profile and lectin activity (A) Bean sample. (B) Protein bands on 12% SDS-PAGE using Coomassie brilliant blue R-250 stain lane profile: 1-crude bean extracts, 2-Ammonium sulphate precipitation (20-50%), 3-Ammonium Sulphate Precipitation (50-90%), 4-PPb-ANF, 5-Marker. (C and D) Erythroagglutinating efficacy of crude, ASP (50-90%), and PPb-ANF. (E) Leucoagglutinating efficacy of PPb-ANF compared with control untreated leucocytes

Table 1: Purification of PPb-ANF from bean sample

Purification stages	Volume in ml	Total protein (mg)	Total HA activity (units)	Specific HA activity (Units/mg)	Recovery in percentage
Crude extract (50% w/v)	500	5290.0	49197.0	9.3	100
Ammonium sulphate precipitation (50-90%saturation rate)	65	732.8	27480.0	37.5	55.8
Post Dialysis and Ultrafiltration by Amicon centrifuge	23	135.8	20370.0	150	41.4

The specific HA activity of PPb-ANF was increased from 9.3 Units/mg (Crude extract) to 150 units/mg (post-dialyzed PPb-ANF) at the purification level. The yield of PPb-ANF was approximately 135.8 mg from 50 gm of bean sample.

Digestive stability of PPb-ANF

PPb-ANF mixed with SGF (including 2500 pepsin activity units/mg test proteins) and incubated at 37 ºC. During incubation time intervals (0, 5, 10, 20, 40, and 60 min), about 200 µl of samples were taken out and placed at 75 ºC to stop the reaction. The aliquots were analyzed by SDS-PAGE 12% gel under a reducing condition. The result shows stability up to 2 h when mixed and incubated with SGF at 37 ºC (fig. 2)

Antioxidant and lipid peroxidation efficacy of PPb-ANF

Interaction of DPPH free radical with an odd electron produced the highest absorption with intense purple color which was read at 517 nm. Free-radical scavenger activity of PPb-ANF. Was determined by reaction with DPPH for 30 min to yield the end product DPPH-H, which is having lower absorbance compared with the standard DPPH due to decreased amount of hydrogen along with the increased number of electrons indicating decolorization from purple to yellow hue. Antioxidant activity of standard ascorbic acid used at 25 µg showed an absorbance of 0.450 at 517 nm with a total of 36.4% of anti-oxidant activity and was compared with an equal concentration of PPb-ANF. Final 400µg of PPb-ANF showed the highest 90% of anti-oxidant activity mentioned in (fig. 2A) Lipid Peroxidation inhibition percentage in 0.5 ml of liver homogenate (Balb/c mouse) in *in vitro* condition were measured by using TBARS (Thiobarbituric acid reacting substances) test using 25-150 µg of PPb-ANF. TBARS is one of the known non-specific methods to measure the extent of oxidative damage by the ROS and release of following by-products of lipid

peroxidation such as aldehydes, alkanes, and isoprostanes. 2-Thiobarbituric acid reacts with one of the important by-products of lipid peroxidation, namely aldehydes-specifically Malondialdehyde (MDA) forming MDA-TBA product Thiobarbituric acid reacting substances (TBARS) which were measured at 532 nm. On increased concentration (25, 50, 100, and 150 µg) of PPb-ANF. Produced increased inhibitory percentage of lipid peroxidation ranging from 58%, 69%, 78%, and 89% were shown in (fig. 2B).

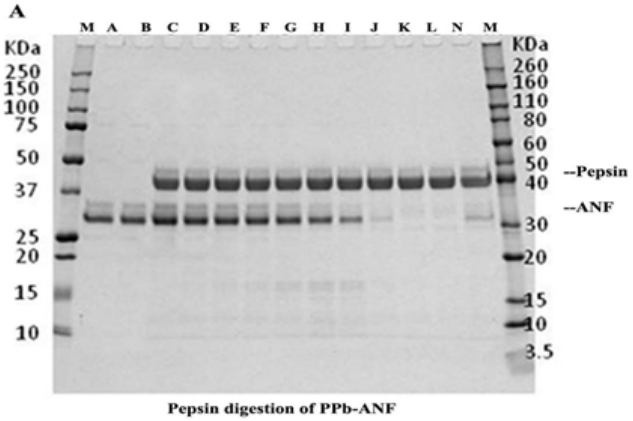

Fig. 2: Pepsin digestion of PPb-ANF-M, marker; A, protein control at time 0 min (P0); B, protein control at time 60 min (P60); C, Digestion control, time 0 min (D0); D, 0.5 min digestion (D0.5); E 2 min digestion (D2); F, 5 min digestion (D5); G, 10 min digestion (D10); H, 20 min digestion (D20); I, 30 min digestion (D30); J, 60 min digestion (D60); K, Pepsin control, time 0 (E0); L, pepsin control time 60 min (E60), N, protein control at 1/10th the target concentration, time 0 (P1/10)

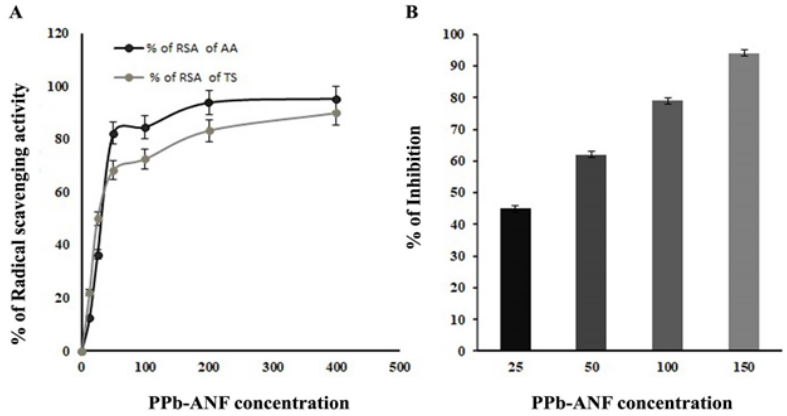

Fig. 3: Antioxidant activity of PPb-ANF: (A) represents the DPPH scavenging activity percentage of PPb-ANF, exhibiting significant radical scavenging activity upon the increasing concentration of PPb-ANF. (Test Sample-TS) and was compared with standard ascorbic acid (AA). (B) PPb-ANF exhibited good inhibitory lipid peroxidation activity with increasing concentration of PPb-ANF and it was mentioned in percentage. Results are the means of three determinations, each conducted in triplicates

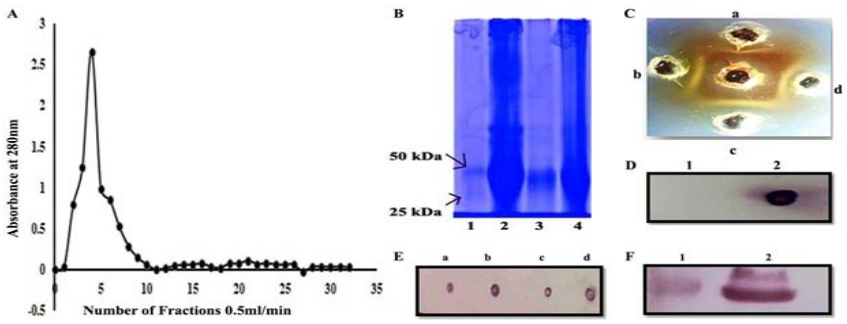

Fig. 4: Purification of anti-PPb-ANF IgG-antibody by Protein-A agarose chromatography: (A) Protein-A affinity chromatography graph for purification fractions of rabbit anti-PPb-ANF antibodies, the first peak was bound peak (IgG), eluted by changing the pH. (B) Gel protein bands (12% gel) using Coomassie brilliant blue under non-reducing condition Lane Profile:-Peak-1 Purified anti-PPb-ANF-IgG fraction, 2-Crude serum sample, 3-Dialyzed serum sample, 4-Ammonium sulfate fraction. (C) Confirmatory assay-ODD (Ouchterlony double diffusion) to analyze the immuno-precipitation of anti-PPb-ANF in different purified steps-a-crude serum, b-20-50% ASP serum, c-50-90% ASP serum, d-Dialyzed serum sample. The precipitation line in an agarose medium indicates specificity against antigen PPb-ANF. (D) Dot-immunoblot analysis for the rabbit anti-PPb-ANF polyclonal antibodies 1. Negative control-BSA and 2. PPb-ANF. (E) Immunoblot analysis of rabbit anti-PPb-ANF polyclonal antibodies. Lane 1-crude bean extract, 2-PPb-ANF

Production and purification of anti-rabbit polyclonal antibody against PPb-ANF

Rabbit-produced anti-PPb-ANF antibodies in immunized anti-sera were collected and purified to homogeneity using ammonium sulfate precipitation, dialysis, and affinity chromatography using protein-A agarose column to obtain a pure anti-PPb-ANF IgG antibody (fig. 3A). The purity of the anti-PPb-ANF antibody fraction was checked by SDS-PAGE in non-reducing conditions and confirmed the presence of antibody by 150 kDa molecular weight band (fig. 3B). PPb-ANF immunized anti-sera was analyzed for the existence of antigen-specific antibodies through confirmatory assays like ODD (fig. 3C), dot-blot (fig. 3D), and immunoblot assays (fig. 3E and F) Rabbit-produced anti-PPb-ANF antibodies showed and confirmed their strong affinity as a precipitation line in dot-blot, the appearance of a band in immunoblot assay. Overall results portray that rabbit-produced and purified serum samples containing IgG-antibody against lectin PPb-ANF showed their potent immunogenic property.

PPb-ANF interaction with natural antibodies (rabbit and healthy human beings)

After showing successful immuno-activity towards anti-rabbit IgG antibody produced in rabbit antisera against PPb-ANF, further lectin PPb-ANF was subjected to antigen-titer, using rabbit immunized and pre-immunized sera in Indirect ELISA assays. Upon increasing the concentration of 5 µg to 5 mg of PPb-ANF, the binding ability of rabbit IgG-antibody was also increased shown (fig. 1A) and was compared with control rabbit pre-immunized sera. A final concentration of 5 mg of PPb-ANF read 0.979 OD to immunized sera, whereas for pre-immunized sera showed a reduced 0.120 OD at 405 nm. Further performed both IgG and IgE-binding ability of PPb-ANF through collected healthy individual serum samples (S1-S7). Obtained IgG, IgE-immuno-reaction. ELISA results were compared with positive control rabbit-produced anti-PPb-ANF IgG antibodies, negative control Bovine serum albumin, and plotted graph. Overall obtained results displayed that PPb-ANF is having potent immunogenic properties (fig. B and C).

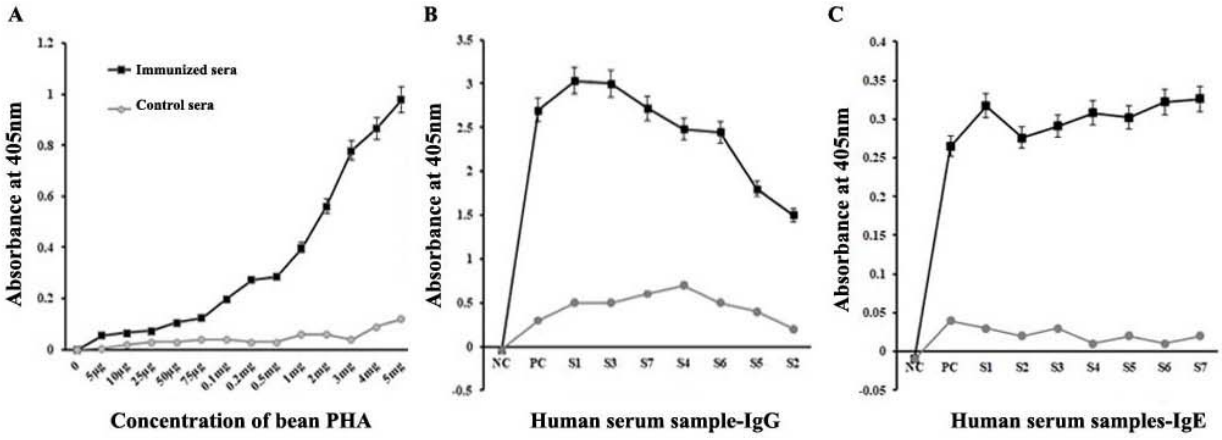

Fig. 5: Rabbit IgG and Human IgG, IgE interaction of PPb-ANF (A) Antigen Titre of PPb-ANF using control pre-immunized and immunized sera were performed using rabbit sera. (B) PPb-ANF showed moderate IgG-binding specificity property in selected human serum samples (C) PPb-ANF showed significant IgE-binding specificity property in human serum samples. Results are the means of three determinations, each conducted in triplicates

Anti-angiogenic parameters of PPb-ANF

Angiogenesis is an important process in all living organisms; in-contrast, metastasis and tumor growth are well dependent on angiogenesis. Thus inhibiting angiogenesis in cancerous conditions resulted in the arrest of tumor growth meanwhile limit the metastatic efficiency of tumors. Anti-angiogenic property of PPb-ANF in Ova CAM assay was assessed. PPb-ANF was found anti-angiogenic, which was ascertained and visualized the inhibition of vascularization; additionally, In-Ova CAM blood vessel counts were drastically decreased. PPb-ANF treated in Ova CAM, showed 70%, and 40% inhibition and inhibited vessel density were visualized (fig. 6).

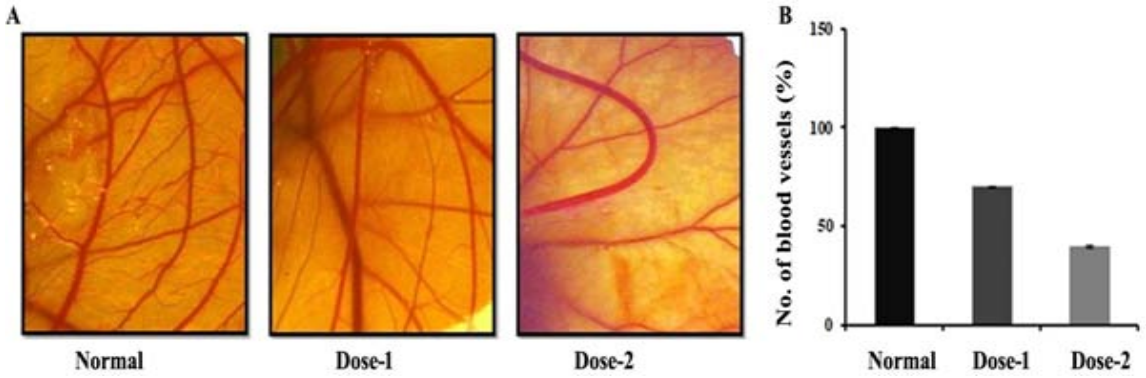

Fig. 6: Anti-angiogenic characteristic of PPb-ANF (A and B) Antiangiogenic activity of PPb-ANF was accessed In-Ova angiogenic assay models. Ova CAM images and graphical images represent the potential anti-angiogenic percentage of PPb-ANF and also observed decreased CAM vessel count density. Upon increasing the concentration of PPb-ANF (dose-1: 1.5µg, dose-2: 3µg) treated CAM models display the inhibition of vessel density in CAM

Anti-cancer efficacy of PPb-ANF

Anticancer properties of PPb-ANF were assessed in BALB/c mice models by injecting Ehrlich ascites carcinoma (EAC) cells on the right thigh along with one control (without EAC cells). The solid tumor model is one of the most valid and representative system of histological types of cancer. PPb-ANF was given at the concentration of 25 mg/kg body weight (Dose 1) and 50 mg/kg (Dose 2) body weight i. p. (Intra-peritoneal) on alternative days of tumor growth. Repeated three trials and obtained results. The anti-tumor activity of PPb-ANF was evaluated in a solid tumor model system; upon treatment with PPb-ANF an indicative reduction in tumor growth volume was observed when differentiated from the untreated mice (fig. 7A). Most specifically, the life span of PPb-ANF treated murine mouse models was three-fold increased when compelled with the untreated mice (fig. 7E). Gross morphological condition of tumors in thigh tissues of both control and treated models were compared and confirmed the drastic regression in the tumor size after treatment with PPb-ANF. Additionally, appearance of treated and control models as well as the morphology of the liver, and spleen, were compared (fig. 7B). Peremptorily, PPb-ANF confirms potent anti-cancer properties.

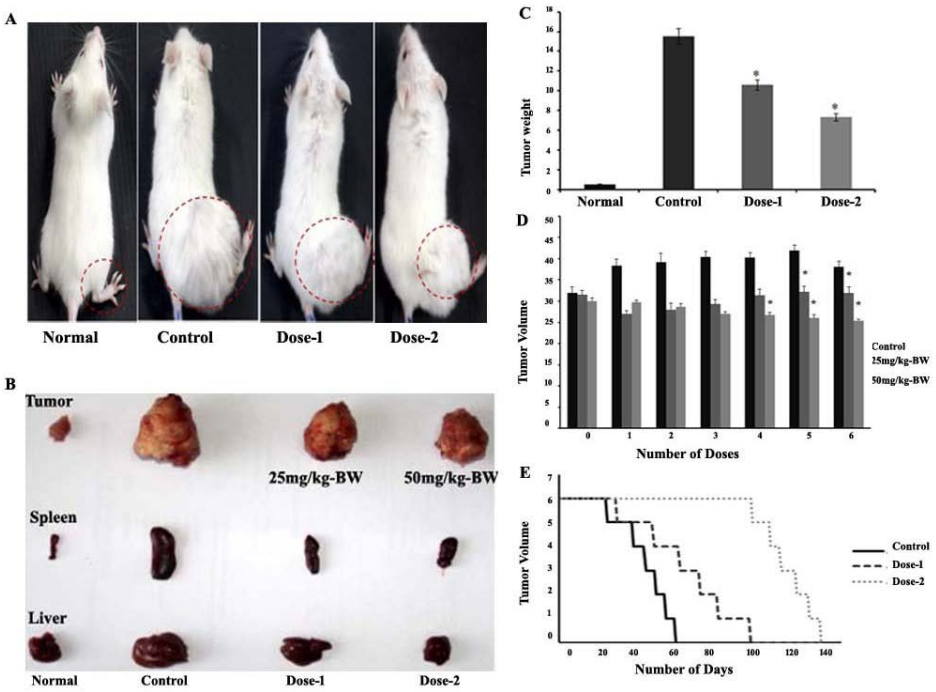

Fig. 7: Effect of PPb-ANF on EAC solid tumor models (A) Morphology of EAC-induced mice models. (B) Gross appearance of excised tumors, spleen, and liver. (C) Effect of PPb-ANF on EAC-induced tumor models exhibited a drastic reduction in tumor weight compared with control tumor model up to 8 gm variance in their size (D) Weight of normal thigh portion, control and PPb-ANF treated tumors in grams. (E) Kaplan-Meier survivability graph curve of animals treated with PPb-ANF exhibited an extended life span of 70 d compared with control mice. Results are the means of three determinations, each conducted in triplicates. Statistically significant values are *p<0.05

DISCUSSION

Legume beans fill a prominent place in human nutrition and are also recognized as a staple food in many countries. With the exception of their inexpensive source of valuable proteins, micronutrients like vitamins and minerals, saccharides, rich dietary fiber, and low-fat contents [18]. Daily intake of legumes contributes to multiplied advantageous physiological effects. Mention, legumes permit the prevention of common metabolic diseases like Diabetic Mellitus, CHD-coronary heart diseases, and also cancer [19]. Especially, Anti-nutritional factors in beans, namely lectins, enzyme inhibitors, and polyphenols are highly biologically active compounds, meanwhile attracting attention as an active preventive agent of metabolic disorders and they are also called “secondary metabolites”. Quite a few of these ANFs have shown themselves as deleterious or propitious effects on both human and animal health, only if it is consumed in adequate amounts [20]. Noticeably most of the secondary metabolites induce dangerous or damaging biological immune responses. Even though some of them are widely adopted for nutrition and also as pharmacologically active agents. Through different metabolism processes of species like digestive process diminution, inactivation of nutrients, metabolic uptake exerts cross-gained optimum nutrition [21]. Most of all ANFs exist in foods are from plant derivation; these ANFs in foods are accountable for the adverse influence on humans if their consumption goes beyond or overreach the maximum limit. Absorption of these ANFs may interrupt the action of absorption of nutrients as well as micronutrients in certain organs. Specifically, their deleterious impact may occur due to the breakdown of certain fragments in these compounds. Despite that, a few of ANFs and their breakdown fragments may induce advantageous health benefits when it exists in limited amounts. The existence of these ANFs in beans exerts both anti-nutritional as well as their beneficial consequences in food. The present research study will provide basic knowledge about the existence of ANF in beans, which this study may use for future findings of new therapeutics [19]. The present study focuses on the existing bean ANF to screen their both beneficial as well as non-beneficial effects. Initially, we prepared a crude extract of a bean sample and checked for the agglutinating capabilities of erythrocytes. Bean crude extract potentially agglutinated erythrocytes; this confirms the existence of ANFs in beans. Further, we subjected for purification of bean-ANFs using 0%-90% ammonium sulfate fractionation. ASP purified bean-ANFs also exerted agglutination of erythrocytes. Crude, and ASP (0-20%; 50-90%) were resolved under 12% SDS-PAGE; PAGE profile displayed the existence of two proteins with the appearance of thick blue bands on the gel. One of the major bands has appeared on 40-54 kDa molecular weight of the protein was known as storage protein [22, 23] and the second small band appeared on 30-32 kDa molecular weight protein and was recognized as bean-ANF. we further continued by subjecting to dialysis of bean-ANF and obtained the partially purified bean-ANF (PPb-ANF). Further, we checked for the agglutination ability of PPb-ANF, which showed potential agglutination capacity over erythro and leucocytes. Through

agglutination of both erythro and leucocytes, PPb-ANF has got specific agglutination properties. PPb-ANF also showed its competitive pepsin digestive stability nature in pepsin digestion. Oxidative stress origination occurred by the imbalance of augmentation and distribution of ROS-reactive oxygen species in cells as well as tissues. Excessive production of ROS in cells can easily cause damage by inducing lipid peroxidation meanwhile also denature the proteins or nucleic acid structures. PPb-ANF showed a significant anti-oxidant property, with increasing concentration by DPPH assay, and was almost 90% efficient when compared with standard ascorbic acid. Similar to the DPPH assay, Lipid peroxidation is one of the mechanisms involved in the damage of cells as well as tissues. Malondialdehyde (MDA) is the end-product, this product was detected by TBARS-thiobarbituric acid reacting substances assay. PPb-ANF showed its potentiality in inhibiting increased lipid peroxidation parameters upon increasing the concentration [24]. PPb-ANF, the immunogenic property was accessed in rabbits and obtained confirmatory assays and immunoblot assays, ELISA assay results were also narrated PPb-ANF as a potent immunogenic agent. PPb-ANF is also showed immunogenic allergenic properties in healthy human serum samples. Hence PPb-ANF also able to interact with both IgG and IgE-antibodies of humans. Neo-vascularization is an essential operation in tumor expansion and is price-marked to be valuable in capillary routing with convoluted as well as distorted and immense vascular branching. Limited oxygen supply phase in tumor cells can upstream trigger the cascade deal further leading to angiogenesis finally deals with the suppression of fast growth of cancer cells [25, 26]. We further accessed to understand the anti-angiogenic property of PPb-ANF using In-Ova-CAM assay. In connection with the PPb-ANF effect on angiogenesis, obtained results indicated that blood vessel counts in Ova CAM were extremely narrowed. PPb-ANF showed its vigorous anti-angiogenic efficacy parameters. Further PPb-ANF was subjected to anti-tumor studies in *in vivo* models using Ehrlich ascites carcinoma cells. Our research studies signified that PPb-ANF remarkably regresses tumor progression [27]. PPb-ANF treated animals showed a drastic decrease in their tumor size upon increasing the concentration of PPb-ANF and meanwhile, their survivability parameters also extended when compared to the control tumor-treated animal model.

CONCLUSION

Overall research study on the hidden anti-nutritional factor in Indian bean with 30-32 kDa molecular weight protein reveals significant antioxidant, immunomodulatory, anti-angiogenic, and anti-cancer properties. Partially purified bean anti-nutritional factor (PPb-ANF) with potent anticancer inhibiting efficacy, anti-angiogenic properties would inaugurate a future-oriented new viewpoint for novel therapeutic agents and also be useful for the development of drugs for various health complications.

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AUTHORS CONTRIBUTIONS

S. N. P planned; H. A. and SD P. conducted all the experiments and prepared the illustrations; S. N. P prepared the initial draft of the manuscript. S. N. P. and B. T. P conceptualized the research, supervised and interpreted the data, and prepared the final version of the manuscript. Final draft preparation, data curation and conception by S. N. P., B. T. P. and N. P. All authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

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