RESEARCH ARTICLE

Co-immunoprecipitation based isolation and identification of Interacting Phytal and Rheumatoid arthritis subject's serum globulins proteins

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Article info:
Received: 17 March 2024
Accepted: 08 May 2024

ABSTRACT

Phytalproteins have an immense role in plant's growth and sustenance processes. They can be used as nutritional enhancers of foods we consume. Rheumatoid arthritis is an autoimmune disorder, prevalent in both sexes of humans and especially those above 50 years. Ayurveda suggests plant oils to reduce the pain and inflammation caused by Rheumatoid arthritis (RA). The current study is based on immunoprecipitation interactions between phytal proteins and gamma globulins of Rheumatoid arthritis subjects to assess their role in diagnostics or as immune modulators. A comparative study of proteins of *Murraya koenigi*, *Basella alba* (leaves), *and Sapindus mukorossi* (seed) has been considered for immunoassays. The protein-protein binding capability assessment has shown a positive response with *Murraya koenigi* phytal proteins than that of *Basella alba*. The globulins of *Sapindus mukorossi* seed proteins indicated more specific and prominent interactions than the previous two. The co-immunoprecipitation bands were subjected to LC/MS followed by Bioinformatic studies based on meta *HawkDock* and PDB sum. Based on the docking study's scores, hydrogen bonds and salt bridges, bonding capability between 10 selected gamma globulin proteins and unique proteins, and 4 phytal proteins of Indian soapberry were established. Q3KNS1(sperm function), unique protein Q7Z351(associated with the immune system) and A0A024617 (Alpha-1-antitrypsin) proteins of human serum showed an interaction with A0A0U1XK40, A0A7H1CQR4 of *Sapindicus*. Studies of RuBISCo (A0A0U1XK40) of Indian soap nut protein and Q3KNS1(Patched domain-containing protein 3 that controls sperm development or sperm function and affects Rheumatoidism of muscles) interactions, can be considered for further studies

Key words: phytal proteins, globulins, Rheumatoid arthritis, immunoassays, binding pattern

Introduction

The autotrophic nature of plants is one of the unique features that raised their position in the biotic world. The numerical applications of plants from primary producers to carbon sinks have extended to medicinal applications. The animal population depends on the plant's secondary metabolites, carbohydrates, pigments, and storage lipids for their survival. Plant proteins are of great importance in the current scenario as the sources of food, nutrition supplements, and immune modulators (Langyan et. al., 2022; Michiel et. al., 2018). The dicotyledonous plants' seeds are often considered as the source of proteins as they help in the germination the development of the plant. Plant-based dietary foods have a good role in controlling Rheumatoid arthritis (Gioia et.al.,2020) as well as medicinal plants such as *Glycyrrhiza glabra, Boswellia serrata* sps an important role in the prevention.(Hajja and Bahlouli,2017)

Immunity is required for the human population to maintain a balanced life and fight against diseases. The immune system in the body identifies the harmful, foreign bodies and initiates a process of fighting by phagocyting the harmful substances using the T cells and B cells. The inability to differentiate the foreign bodies from that of the own immune cells results in autoimmune disorders. The most prevalent such autoimmune disorder is Rheumatoid Arthritis which affects females above 50 years of age more than other subjects. Several studies have been carried out to identify the reasons for this health hazard, characterize the prevailing conditions, control the disorder, and diagnose the

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disease. The protein expression patterns have gained more prominence as the unique proteins and the related metabolic pathways play valuable role in diagnostics and help in preventive medicine. Topical applications are often used to control inflammation, and pain and of temporary in nature. Very few studies highlight the identification of proteins from the profile studies and relate their importance in diagnostics and disease control.

Protein-protein interaction (PPI) studies help to understand the protein function and binding level, based on affinity purification coupled mass spectrometry (AP/MS) (Michiel Bontinck et al., 2018). PPI studies also play an important role to assess tissue-specific interactions during developmental stages, immune-specific studies, and, protein and surface membrane protein/ glycoprotein or lipoprotein interactions. Therapeutically active proteins extracted from medicinal plants have a promising role in the control of human diseases as they exhibited anti-oxidant, anti-cancerous, and neuro-modulatory functions to name a few(Snober S.Wani et.al.,2020). Plants commonly used for consumption purposes such as vegetables and detergent are considered to study plant proteins. Immunoprecipitation of intact protein complexes is known as Co-Immunoprecipation, which could pull the entire protein complex out of solution and thereby identify unknown members of the complex. Co-IP is a powerful technique that is used regularly by molecular biologists to analyze protein–protein interactions (Lin and Lai2018). Three plants were selected to study plant proteins of glabrous climber used as a vegetable (*Basella alba*), an aromatic cuisine flavor enhancer (*Murayya koienigi*), and, seeds of Indian soapberry (*Sapindus mukorossi*). Plant proteins were extracted from the leaves of *Basella alba*, *Murayya koienigi*, and, seeds of Indian soapberry (*Sapindus mukorossi*). The current study is aimed at understanding the interactions and assessing the binding pattern with RA antibodies based on immune assays. LC-MS and bioinformatics-based analyses to identify the proteins and characterize which can be of diagnostic or curative purposes.

Materials and Methods

Serum sample collection

Serum samples of subjects diagnosed with RA based on American College of Rheumatism (ACR) and the European League Against Rheumatism (EULAR) criteria considering serological tests and phase reactant testing. The samples with a total score ≥ 6 were collected from Shah pathology laboratory. Pooled serum samples in triplicates were considered for the analyses.

Serum Slide electrophoresis

This technique was used to separate gamma globulin that is higher in level in RA and most significantly contains immunoglobulins. So, 1% agar gel is prepared in Tris glycine buffer and 6 ml of the solution is spread on a slide. 20 µl of serum sample was mixed with loading dye (bromophenol blue) loaded onto the slide using a coverslip. The slide is electrophoresed in the horizontal gel electrophoresis apparatus which is filled with Tris Glycine buffer and that runs for 10-15 minutes at 150 V. (Mayer and Walker,1987). Gamma globulin band was cut from the gel and homogenized with 1-2ml of pbs buffer and centrifuged at 2000 rpm for 5 min. The globulins thus collected were used from immunoprecipitation assays.

Plant material

Plants commonly used for daily consumption in normal households as vegetables and detergent are considered for the current study. Three plants were selected to study plant proteins a.glabrous climber used as a vegetable Malabar spinach *(Basella alba*), b.an aromatic cuisine flavour enhancer - kadipatta (*Murayya koienigi*), and seeds of Indian soapberry (*Sapindus mukorossi*) and identified by the Botany Department, Mithibai College. The leaves of Malabar spinach and kadipatta were collected from the local market and, cotyledons of Indian soapberry were collected by breaking hard skinned shells of seeds (Fig.1).

Protein extraction

Alkaline Extraction and Isoelectric Precipitation (Soo et.al.2021) method was used to extract protein from leaves of kadi patta (*Murayya)* and Malabar spinach (*Basal*). For this procedure, the leaves were washed with distilled water 2-3 times and then dried in the shade for 8 days. The leaves were ground into a fine powder using a mortar and pestle. Thirty

Figure 1. *3 selected plants for precipitation studies. (B-Basal alba (Malabar spinach), S-Sapindus mukossoki- Indian soap berry, M-Murayya koeinigi – Curry leaf)*

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grams of this powder was homogenized with 20 ml of 0.1 N NaOH and incubated at room temperature for 30 minutes. This solution was filtered using Whatman filter paper No.1 paper and pH was checked. The filtered solution of pH 7.8 was brought to pH 4.0 using 0.1N HCl and, centrifuged at 3500 rpm for 10 minutes at a low temperature (4°C). The supernatant was discarded, and the precipitate was mixed with PBS and stored for future use.

Extraction of proteins from seeds of Indian soapberry was performed using both sequential fractionation methods (Givonetti et. al., 2021) and as well as alkaline extraction and isoelectric precipitation methods. 50 grams of seeds were broken down to isolate the cotyledons of 13.685gm to extract proteins. The cotyledon sample was divided into two equal parts of 6.843 gm. One part (A) was homogenized with NaOH and put in a centrifuge. After centrifugation at 500 rpm for 20 minutes the supernatant was collected and 0.1N HCl was added till the precipitation was observed. The precipitate was collected and filtered. The other part (B) of 6.842 g of sample was homogenized using 35 ml Hexane and was kept on an orbital rotary shaker overnight. Hexane was removed by air drying the seed content in a clean grease free petri plate. The sample was further divided into four parts to extract Albumins, Globulins, Prolamines and, Gluteins. Each sample was further treated with 20ml each of double distilled water or 5% Nal solution or 4:1 ethanol or Mercaptoethanol mixture and 0.1NNaOH solutions followed by vortexing for 5 min. each. After incubation, the tubes were centrifuged for 25 min at 500 rpm to extract Albumins, Globulin, Prolamins, and Gluteins respectively.

Estimation of Protein concentration

Biuret method was considered for qualitative and quantitative study of proteins using BSA as standard. Standard solutions were prepared in the range of 0.5 to 15 g/l from the stock solution. 1ml of sample and standards were taken in separate tubes and added with 4 ml of biuret solution. After 10 minutes of incubation, the absorbance was taken at 660 nm. The concentration of plant proteins was extrapolated from the standard graph of BSA solutions vs absorbance at 660 nm (Nowotny, 1979).

Immunoprecipitation assays

A double immunodiffusion assay was done to identify the protein (antigen) of which plant was binding with the proteins (antibodies) present in serum. 1% agar prepared in Borate Buffer was considered to prepare the slide and left to dry for 3-5 minutes. The gel was punched with a borer and 15 microliters of each protein and serum sample were loaded in the wells. The slide was incubated for 2 and 4 hours under moist conditions at 37^{O}Cand the slide was fixed with 0.05% sodium azide for 5 minutes. Followed by staining with CBB stain and destaining (Mayer and Walker,1987).

Radial immunodiffusion assay

This technique was also performed to study the binding pattern of the antibodies present in the serum with the antigens that may be present in the protein extracted from the plants. 1% agar prepared in Borate Buffer was prepared to which 50-100µl of the serum was added prior to spreading it on the glass slide. Spread on a glass slide and left to dry, wells were punched and 15 microliters of each protein were loaded. The slide was incubated for 2 and 4 hours under moist conditions at 37°C and the slide was fixed with 0.05% sodium azide for 5 minutes. Followed by staining with CBB stain and destaining. (Mayer and Walker, 1987)

Rocket immuno-electrophoresis

Gamma globulin separated from the serum sample is mixed with 1.5% agar in Tris-glycine buffer spread on a glass slide and 1mm wells are bored into the gel. Crude protein samples of all three plants and globulin protein samples of *Sapindus mukorossi* seeds were dialyzed using a suitable dialysis buffer. Each sample was mixed with loading dye (Bromophenol Blue) in the ratio of 3:1 (15 µl of the sample and 5 µl of loading dye) and loaded in each well. The slide is electrophoresed for 15min. at 150 V. (Mayer and Walker,1987)

Co-immunoprecipitation (Co-IP) assays

Molecular Biologists use Co-IP, a powerful technique to analyze protein–protein interactions. In this technique, intact protein complexes are considered, which could pull the entire protein complex out of the solution and thereby identify unknown members of the complex. The earlier studies considered affinity chromatography/Mass spectrometry for the co-immunoprecipitation assays (Lin and Lai, 2017) The current study is based on LC/MS analysis of the complex extract from the immunoprecipitation bands considered to identify the proteins (Ming et.al.,2020). Excised gel pieces of the precipitation bands were de-stained and incubated at 37ºC. Overnight procedures comprising reduction, alkylation, and digestion procedures were performed further. In-Gel Tryptic Digestion Kit protocol (Thermo-Fisher Scientific) was considered along with mass spectrometric study

LC-MS/MS: Thermo EASY-Nlc 1000 UPLC system was used to analyze the sample. The Q Exactive TM Quadrupole-Orbitrap instrument (Thermo Scientific, Waltham, Massachusetts, USA) was used. During the study, pre-column Acclaim PepMap 100, 100um x 2cm nano viper, and analytical column PepMap RSLC C18, 2 um; 100 A x 50 cm were considered to perform the analyses. solvent A (0.1% FA in milliq water) and, solvent B: [85:15 (ACN:milliq water) + 0.1% FA]were used as the mobile phase solvents. The scan procedure was carried out at a range of 350–2000 m/z, and processed at 70,000 resolutions in Orbitrap for 120

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minutes with a constant flow rate of 3.0 l/min. Data analysis software such as Thermo Proteome Discoverer 2.2 and, UniProt Databases of *Sapindus* and *Homo sapiens* are used for the identification of proteins.

In silico analyses of Protein-Protein interactions

The plant and gamma globulin proteins involved in the protein-protein interaction (PPI) were identified using protein docking studies. The proteins identified from the co-immuno precipitation band were identified based on *Homo sapiens* database and *Sapindus* database. These were studied using Metascape software for their reactome and genome functions. (Zhou et.al., 2019). Further, the gamma globulin and phytal proteins associations were identified and assessed using bioinformatics tools such as *HawkDock*, GRAMM-S, PDB sum software. Based on the reaction scores, most important interacting proteins were identified. (Weng et. al., 2019; Laskowiski et. al., 2018)

Results

The serum protein electrophoresis resulted in separation of 5 bands of proteins based on their net charge, size, and shape on the slide. The gamma Globulins albumin found close to negative electrode. The gamma globulins band was cut and homogenized in PBS buffer (pH7.4). The homogenate is centrifuged at 2000 rpm for 5 min. and supernatant was collected. The separated gamma globulins from the Rheumatoid arthritis patient's serum were considered for immunoassays

Proteins were extracted from the leaves and seeds of dicotyledon plants such as Malabar Spinach, curry leaf, and Indian soapberry using the standard methods. The quantitative analyses of extracted proteins of *Sapindus* by Biuret method represented for 100gm of seeds are Albumin- 1.28gm, Globulin- 1.44gm, Glutelin: 1.60gm, and Prolamine: 1.51gm. The highest concentration was glutelin (1.60gm) followed by Prolamine, Globulin, and Albumin.

The leaf proteins of *Basella alba* and *Murayya koenigi* indicated a good clearance of the zone representing effective precipitation against the gamma globulins. (Fig.2a, 2b). A comparatively faint precipitin bands were seen in the case of *Basella alba*, *Murayya koenigi* when compared to *Sapindus sp. Basella alba* (Malabar Spinach) and *Murraya koenigii* (Curry Leaves) showed greater reaction with radial immunoassay and also double immuno-diffusion.

In the case of *Sapindus* the crude proteins as well as the four extracted fractions of Albumin, Globulin, Glutelin and Prolamine were considered for immunoassays. The globulin

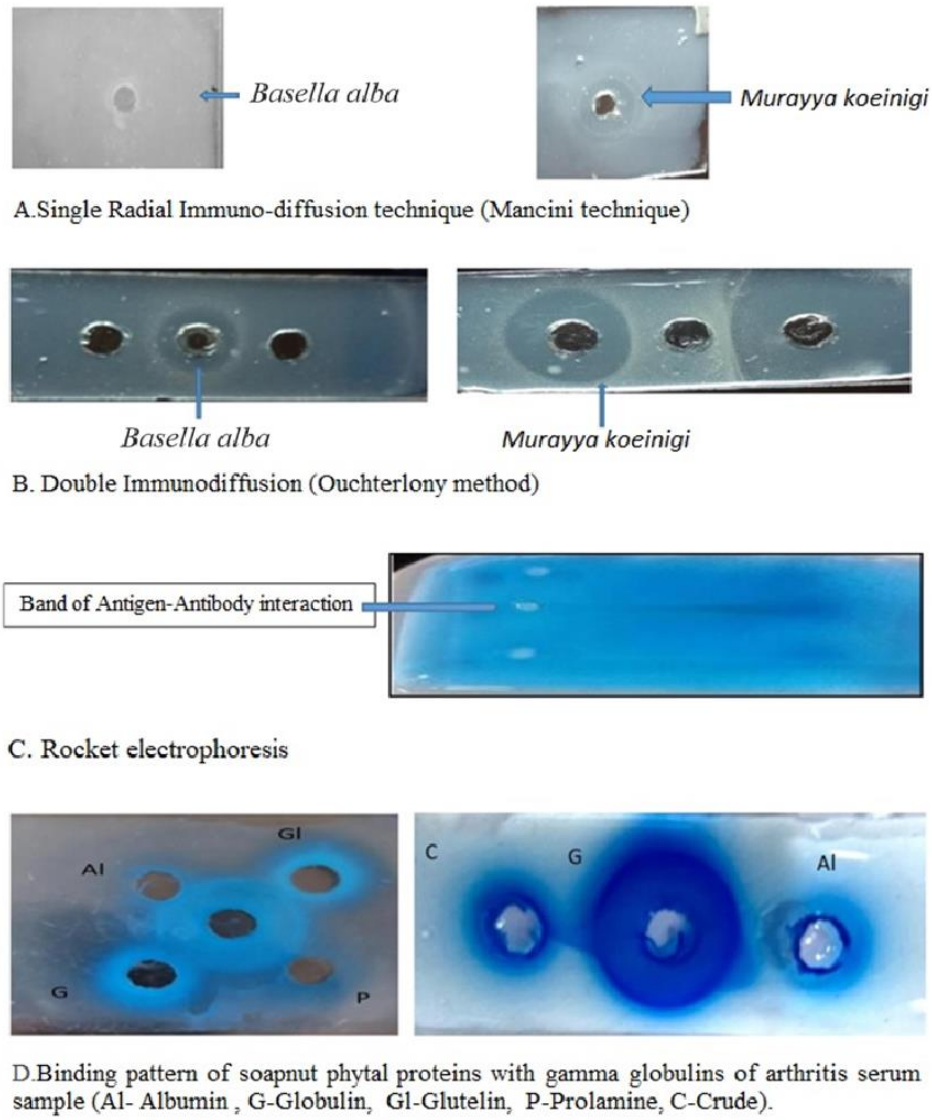

Figure 2. *Plant leaves Protein extract activity with Globulin band from patients serum sample.*

DOI: 10.69085/jbb20241061

proteins of the Indian soapberry seed have shown maximum reaction region and a prominent precipitant band. (Fig 2D). Out of all 3 plants considered for research purpose globulins of *Sapindus mukorossi* (Indian soapberry/soapnut) has a better antigen-antibody binding reaction with the gamma globulin of human serum among all.

The best results were expressed in the globulin and gamma globulin interactions. The precipitation band of these reactions was further analyzed using the LC-MS analysis (Fig. 3a,b). The spectrum of bands of proteins was characterized and identified using bioinformatics tools. Soft wares such as Thermo Proteome Discoverer 2.2 for Data

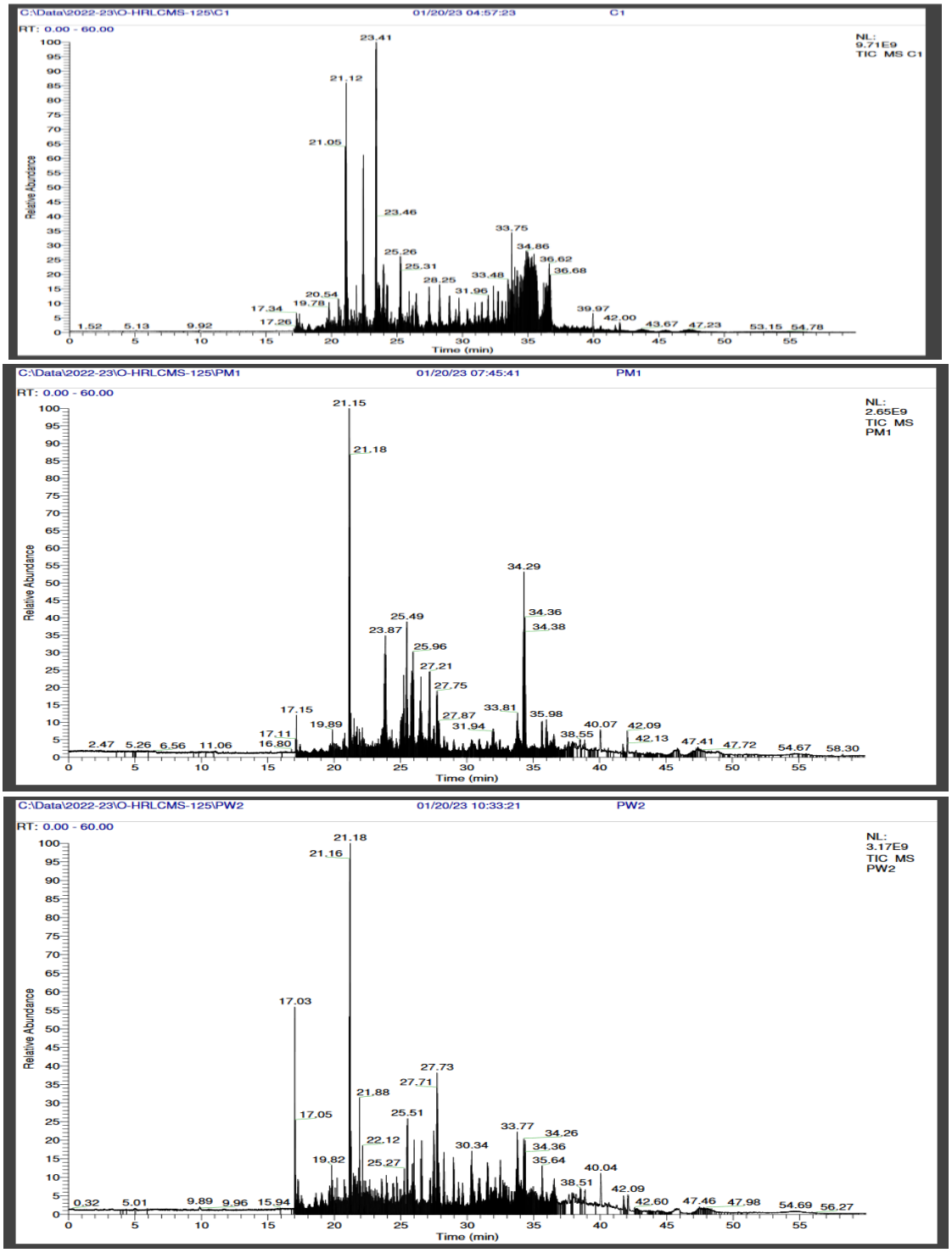

Figure 3. *LC-MS Analysis of Co-Immunoprecipitin band of Phytal proteins of Indian soapberry and serum globulins of Rheumatoid arthritis subjects.*

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analysis and, UniProt Databases of plant (*Sapindus*) and *Homo sapiens* used for the identification of proteins in the bands. Bioinformatic tools opened a new arena of interest to identify the proteins involved and their possible role in the diagnostics/ analysis /control of rheumatoid arthritis.

Altogether 30 proteins were identified in the gamma globulin and 7 of Sapindus plant origin in the protein precipitation band. Of the 30 proteins identified from the human database, 24 were represented by their genes and considered for the Metascape analysis while 4 were unique proteins with Ig G-like domains and without any representative genes and 2 virus-like proteins.

Based on the Metascape analysis, the list of 24 genes can be categorized into 5 sets indicating their role in the regulation of protein stability, Neutrophil degranulation, the killing of cells of another organism, retina homeostasis, and intermediate filament organization. The gene ontology biological processes represented biological regulation,

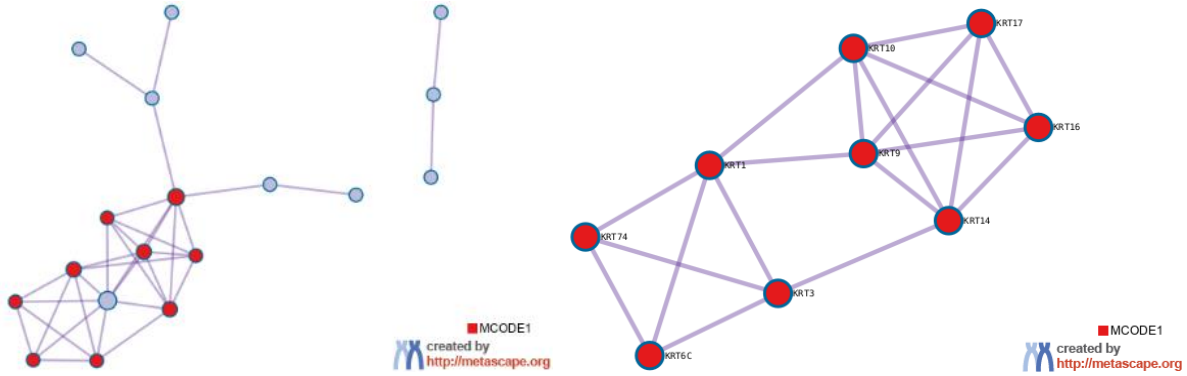

Figure 4a. *Protein-protein interaction network and MCODE components identified in the gene lists.*

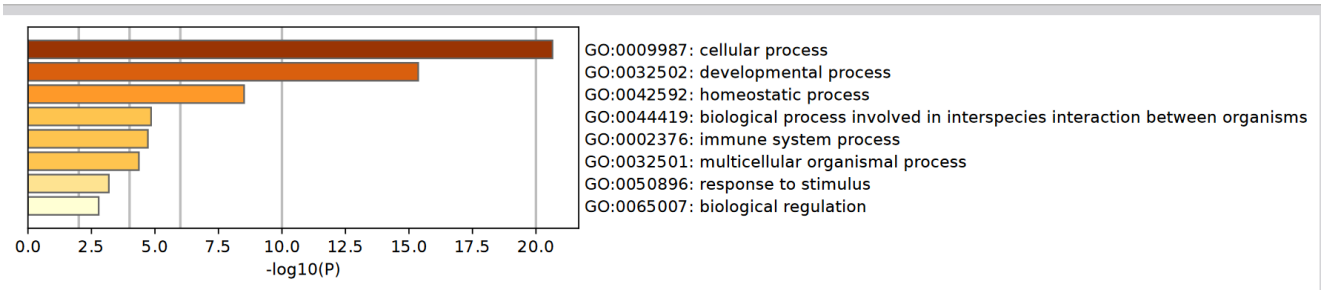

Figure 4b. *Enriched Ontology Clusters.*

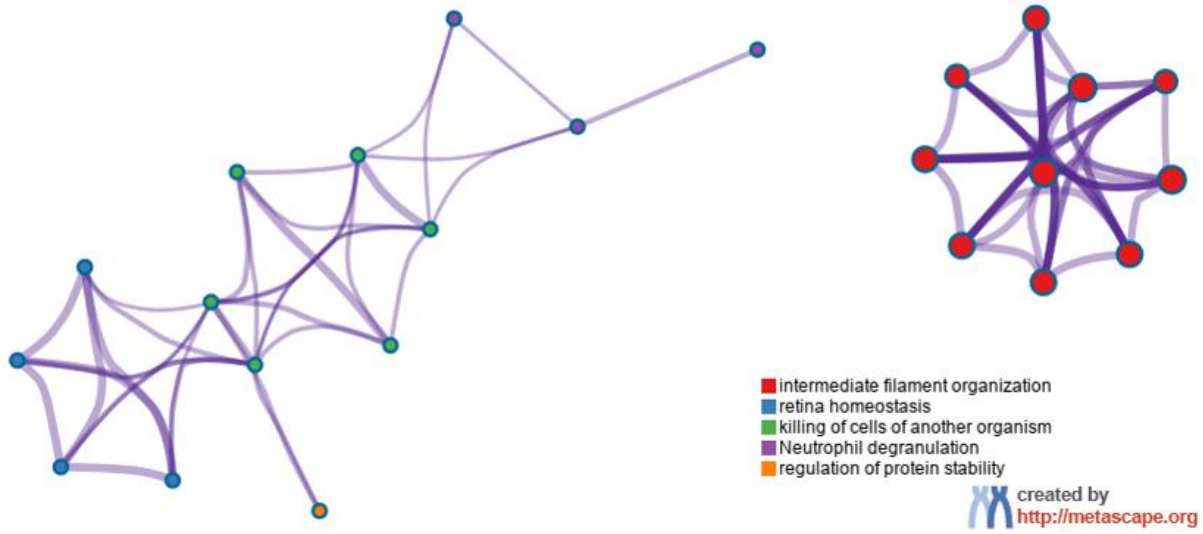

Figure 4b. *Enriched ontology clusters (coloured by cluster ID).*

response to stimulus, multicellular organismal process, immune system process, biological process involved in interspecies interaction between organisms, homeostatic process, developmental process, and, cellular process. Pathway and process enrichment analysis has been carried out with each gene set using ontology sources such as KEGG Pathway, GO Biological Processes, Reactome Gene Sets, Canonical Pathways, CORUM, Wiki Pathways, and PANTHER Pathway revealed 4 GO biological processes (intermediate filament organization, retina homeostasis, killing of cells of another organism, Regulation of protein stability) and 1 Reactome gene set of Neutrophil degranulation. A network plot was created in terms of similarity > 0.3 and p-values from each of the 20 clusters. The networks can be visualized using Cytoscape (Fig.4a,b)

Protein-protein interaction enrichment analysis has been carried out among the human genes listed and Molecular Complex Detection (MCODE) algorithm was applied to identify densely connected network components. The three best-scoring terms based on p-value have been considered for the functional description of the corresponding components. (Fig c).

The different enrichment analyses described the pathway and process cell type signatures, the relation of genes with diseases (DisGeNET) tissue-specific functions (PaGenBase) as well as the transcription targets (Table-1). CSHL1 and TATA-1 are the two transcription-based targets enrichment areas comprising C3 as Regulatory Target and (TFTs) Transcription Factor Targets (TFT:GTRD or TFT_LEGACY) .

The current study indicated the presence of Reactome-based genes especially represented the neutrophil degranulation process. Neutrophils are considered heroes of the immune system as they infiltrate sites of inflammation and behave as innate and adaptive immune response regulators. The pathogenesis of various system autoimmune diseases is due to neutrophils and aberrant neutrophil cell death (Mariana J Kaplan 2013). Besides taking a role in pathogen elimination, degranulation, and phagocytosis, they also possess antigen-presenting capabilities, produce cytokines and chemokines as well unleash immunomodulatory functions in the case of autoimmune

Table 1. *Metascape Annotation of genes*

Protein	id	Description	Biological process(GO)	Protein function	Subcellular location
KRT1	3848	keratin 1	GO:0001867 complement activation, lectin pathway;GO:0051290 protein heterotetramerization;GO:0042730 fibrinolysis	Human disease related genes:Congenital malformations:Congenital malformations of skin; Predicted intracellular proteins;	
KRT10	3858	keratin 10	GO:0051290,91 protein heterotetramerization; protein heterooligomerization;GO:0045684 positive regulation of epidermis development	Predicted secreted proteins; Human disease related genes:Congenital malformations:Congenital malformations of skin; Predicted intracellular proteins	
KRT2	3849	keratin 2	GO:0032980 keratinocyte activation;GO:0051546 keratinocyte migration;GO:0003334 keratinocyte development	Human disease related genes:Congenital malformations:Congenital malformations of skin; FDA approved drug targets:Small molecule drugs; Predicted intracellular proteins	
KRT9	3857	keratin 9	GO:0045109 intermediate filament organization;GO:0045104 intermediate filament cytoskeleton organization;GO:0045103 intermediate filament-based process	Human disease related genes:Congenital malformations:Congenital malformations of skin; Predicted intracellular proteins	
KRT6E	286887	keratin 6C	GO:0045109 intermediate filament organization;GO:0031424 keratinization;GO:0045104 intermediate filament cytoskeleton organization	Human disease related genes:Congenital malformations:Congenital malformations of skin; Predicted intracellular proteins	Intermediate filaments (Approved)

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Table 1 (Cont'd). *Metascape Annotation of genes*

Protein	id	Description	Biological process(GO)	Protein function	Subcellular location
KRT16	3868	keratin 16	GO:0051546 keratinocyte migration;GO:0061436 establishment of skin barrier;GO:0045109 intermediate filament organization	Human disease related genes:Congenital malformations:Congenital malformations of skin; Predicted intracellular proteins	Intermediate filaments (Approved)
KRT15	3866	keratin 15	GO:0045109 intermediate filament organization;GO:0045104 intermediate filament cytoskeleton organization;GO:0045103 intermediate filament-based process	Cancer-related genes:Candidate cancer biomarkers; Predicted intracellular proteins	Intermediate filaments (Supported); Additional: Nucleoplasm
KRT5	3852	keratin 5	GO:0045107 intermediate filament polymerization;GO:0045105 intermediate filament polymerization or depolymerization;GO:0045109 intermediate filament organization	Human disease related genes:Skin diseases:Skin and soft tissue diseases; Human disease related genes:Congenital malformations:Congenital malformations of skin; Predicted intracellular proteins	Intermediate filaments (Approved)
KRT17	3872	keratin 17	GO:0051798 positive regulation of hair follicle development;GO:0051797 regulation of hair follicle development;GO:0042634 regulation of hair cycle	Human disease related genes:Congenital malformations:Congenital malformations of skin; Cancer-related genes:Candidate cancer biomarkers; Predicted intracellular proteins; Disease related genes	Intermediate filaments (Enhanced)
TF	7018	transferrin	GO:0034756 regulation of iron ion transport;GO:0071281 cellular response to iron ion;GO:0045780 positive regulation of bone resorption	Disease related genes; Human disease related genes:Cardiovascular diseases:Hematologic diseases; Predicted secreted proteins; Cancer-related genes:Candidate cancer biomarkers; Predicted intracellular proteins	
DCD	117159	dermcidin	GO:0051873 killing by host of symbiont cells;GO:0050832 defense response to fungus;GO:0009620 response to fungus	Predicted secreted proteins; Transporters:Transporter channels and pores	
DSG1	1828	desmoglein 1	GO:0032570 response to progesterone;GO:0016339 calcium-dependent cell-cell adhesion via plasma membrane cell adhesion molecules;GO:0060135 maternal process involved in female pregnancy	Human disease related genes:Congenital malformations:Congenital malformations of skin; Disease related genes	

Table 1 (Cont'd). *Metascape Annotation of genes*

Protein	id	Description	Biological process(GO)	Protein function	Subcellular location
SERPINA1	5265	serpin family A member 1	GO:0006953 acute-phase response;GO:0002526 acute inflammatory response;GO:0010951 negative regulation of endopeptidase activity	Disease related genes; Candidate cardiovascular disease genes; Predicted secreted proteins; Cancer-related genes:Candidate cancer biomarkers; Predicted intracellular proteins; Human disease related genes:Respiratory diseases:Lung diseases; Human disease related genes:Congenital disorders of metabolism:Other congenital disorders of metabolism	Vesicles (Enhanced)
LYZ	4069	lysozyme	GO:0031640 killing of cells of another organism;GO:0001895 retina homeostasis;GO:0050829 defense response to Gram-negative bacterium	ENZYME proteins:Hydrolases; Disease related genes; Enzymes; Potential drug targets; Predicted secreted proteins; Human disease related genes:Nervous system diseases:Neurodegenerative diseases	Actin filaments;Golgi apparatus (Approved); Additional: Nucleoplasm
HP	3240	haptoglobin	GO:2000296 negative regulation of hydrogen peroxide catabolic process;GO:2000295 regulation of hydrogen peroxide catabolic process;GO:0010727 negative regulation of hydrogen peroxide metabolic process	Disease related genes; Enzymes; Peptidases:Serine-type peptidases; Potential drug targets; Predicted secreted proteins; Cancer-related genes:Candidate cancer biomarkers; Predicted intracellular proteins	Vesicles (Approved)
PIP	5304	prolactin induced protein	GO:0070233 negative regulation of T cell apoptotic process;GO:0070229 negative regulation of lymphocyte apoptotic process;GO:0070232 regulation of T cell apoptotic process	Predicted secreted proteins	
HEL112	6233	ribosomal protein S27a	GO:0002181 cytoplasmic translation;GO:0006412 translation;GO:0043043 peptide biosynthetic process	Ribosomal proteins; Predicted intracellular proteins	Cytosol;Endoplasmic reticulum;Nucleoli (Supported)
GAPDH	2597	glyceraldehyde-3-phosphate dehydrogenase	GO:0035606 peptidyl-cysteine S-trans-nitrosylation;GO:0017014 protein nitrosylation;GO:0018119 peptidyl-cysteine S-nitrosylation	ENZYME proteins:Oxidoreductases; Enzymes; FDA approved drug targets:Small molecule drugs; Predicted intracellular proteins	Cytosol;Plasma membrane (Enhanced); Additional: Nuclear membrane;Vesicles

Table 1 (Cont'd). *Metascape Annotation of genes*

Protein	id	Description	Biological process(GO)	Protein function	Subcellular location
POTEF	728378	POTE ankyrin domain family member F	GO:0098974 postsynaptic actin cytoskeleton organization;GO:0099188 postsynaptic cytoskeleton organization;GO:0001895 retina homeostasis	Cancer-related genes:Mutational cancer driver genes; Predicted intracellular proteins	
KRT74	121391	keratin 74	GO:0045109 intermediate filament organization;GO:0031424 keratinization;GO:0045104 intermediate filament cytoskeleton organization	Human disease related genes:Skin diseases:Skin and soft tissue diseases; Human disease related genes:Congenital malformations:Congenital malformations of skin; Predicted intracellular proteins; Disease related genes	Intermediate filaments (Approved); Additional: Cytosol;Nucleoli
PTHD3		None	None	None	None
ZNF278	23598	POZ/BTB and AT hook containing zinc finger 1	GO:0010596 negative regulation of endothelial cell migration;GO:0010633 negative regulation of epithelial cell migration;GO:0008584 male gonad development	Transcription factors:Zinc-coordinating DNA-binding domains; Predicted intracellular proteins; Cancer-related genes; Disease related genes	Nucleoplasm (Supported)

diseases (Samal Bissenove, 2022). Certain of the mechanisms such as neutrophil extracellular trap (NET) formation and the release of neutrophil nuclear and cytoplasmic contents (NETosis) lead to inflammation which can be a source of citrullinated antigens in RA (Lingshu Zhang, 2022). Especially the dysregulated neutrophil activation contributes to the onset of autoimmune diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus.

The list of proteins observed along with the genes were A0A024R6I7(Alpha-1-antitrypsin, gene-SERPINA1) ; H0Y300(Haptoglobin, gene-HP); H6VRG1(Keratin, type II cytoskeletal 1, gene-KRT1); P02787(Serotransferrin, gene-TF); P04406 (Glyceraldehyde-3-phosphate dehydrogenase, gene-GAPDH); P08779 (Keratin, type I cytoskeletal 16, gene-KRT16); Q02413 (Desmoglein-1, gene-DSG1); P61626 (Lysozyme C, gene –LYZ) in the co-immunoprecipitation protein band along with *Sapindus* phytal proteins.

Ig-like domain-containing protein (Q8TCD0), Keratin, type I cytoskeletal 17 (Q04695), Uncharacterized protein DKFZp686K18196 (Q6N092), MS-D3 heavy chain variable region (A0A0X9T7T4), and, Patched domain-containing protein 3 (Q3KNS1), A0A024R6I7 (Alpha1 Anti-Trypsin protein) and HOY300 (Haptoglobin) along with 3 reported Unique proteins represented by B2RDW1, Q6DHW4, and Q7Z35 of the 13 exclusive gamma globulin proteins of human serum of the immunoprecipitation band were considered for the interaction assessment.

Around 7 proteins represented the globulins of *Sapindus* viz (A0A0U1XK40) Ribulose bisphosphate carboxylase large chain, (A0A7H1CQR4) ATP synthase subunit alpha, (A0A0K1NZN7) Structural maintenance of chromosomes protein 1 (Fragment), (A0A0U1XGS5) ATP synthase subunit beta, chloroplastic, (A0A0K1P088) DNA mismatch repair protein (Fragment), (A0A0K1NZV4) Structural maintenance of chromosomes protein 2 (Fragment), and (A0A6M6R932) Genome polyprotein.

Characterization of globulin proteins of RA patient's serum sample,

1. Ig-like domain-containing protein(Q8TCD0): 293 AA. Reacts with LRRK2 protein produced due to as well as with *Homo sapiens* 293 cells transformed with SV40 large T antigen by having an interaction.

2. Keratin, type I cytoskeletal 17(Q04695): 432 AA. The protein is involved in tissue repair, acts as a promoter of epithelial proliferation, regulates the immune response in skin, and promotes Th1/Th17-dominated immune environment. The studies indicated that it has a role as an auto-antigen in the immune pathogenesis of psoriasis, and certain peptide regions behave as major targets for autoreactive T-cells. The variation in these proteins may lead to disease.

3. Uncharacterized protein DKFZp686K18196 (Q6N092): 519 AA. has four Ig-like domains

4. MS-D3 heavy chain variable region (A0A0X9T7T4): 134 AA, immunoglobin heavy chain

5. Patched domain-containing protein 3 (Q3KNS1): 954AA with a function of the key role in sperm development or sperm function but not in spermatogenesis or male fertility.

6. Alpha-1-antitrypsin (A0A024R6I7): represented by 418 AA and Gene SERPINA1. Its molecular functions include hydrolase activity and catalase activity along with regulatory activity especially serine-type endopeptidase inhibitor activity.

7. H0Y300: represents the protein Haptoglobin, represented by the HP gene. It has 442 AA and shows catalytic activity on proteins and at molecular level serine–type endopeptidase activity.

Unique proteins:

1.B2RDW1: commonly known as Ubiquitin-40S ribosomal protein S27 or Ubiquitin carboxyl extension protein 80. It is a Ribosomal protein and Signal recognition particle The molecular function is. The KEG database indicated its role in Mitophagy where in, it removes damaged mitochondria. Previous studies indicated that thedysregulated

mitophagy implicates numerous autoimmune diseases (TNF-α Induces Mitophagy in Rheumatoid Arthritis)(Ji et. al., 2022).

2.Q6DHW4: A unique protein with an Ig-like domain. It is found in primary B- cells with unspecified role and function. 20-237 AA chain represents Ig-like domain-containing protein which are often involved in cell-cell recognition, cell-surface receptors, muscle structure the immune system especially responsible for cell-mediated immune responses.

3.Q7Z351: Known as an Uncharacterized protein DKFZp 686N02209. A unique protein with 487 amino acids with different functions represented by DKFZp 686N02209 gene. Has 4 Ig-like domains at amino acid regions of 21-125,158-251, 273-372, and 381-477. Most of the Ig-like domains are involved in a variety of functions such as cell-cell recognition, cell-surface receptors, muscle structure, and the immune system. An Ig-like fold can be found in diverse proteins as well as immunoglobulin molecules. Ig-like domains occur in receptors such as various T-cell antigen receptors, several cell adhesion molecules, MHC class I and II antigens, as well as the hemolin, and titin, telokin and twitching (muscle proteins).

Sapindus representative seed proteins:

1. Ribulose bisphosphate carboxylase large chain (A0A0U1XK40): It has catalytic activity. The two catalytic reactions are the carboxylation of D-ribulose 1,5-bisphosphate, a primary event in carbon dioxide fixation. The other is the oxidative fragmentation of the pentose substrate in the photorespiration process. Both reactions occur simultaneously and in competition at the same active site. It has 1 co-factor binding site and an active site for proton transport.

2. ATP synthase subunit alpha (A0A7H1CQR4): Its biological functions include -ATP synthesis, Hydrogen ion transport, Ion transport, and Transport. Its other function is the capability of ATP-binding and Nucleotide-binding as a ligand.

3. Structural maintenance of chromosomes protein 1 (Fragment) (A0A0K1NZN7): has 1,047 AA hence not considered for docking studies.

4. ATP synthase subunit beta, chloroplastic (A0A0U1XGS5): It is 498 AAsequence , Produces ATP from ADP in the presence of a proton gradient across the membrane. The catalytic sites are hosted primarily by the beta subunits. Its molecular functions include ATP binding, proton-transporting ATP synthase activity, and proton-transporting ATPase activity

5. DNA mismatch repair protein (Fragment) (A0A0K1P088): 616AA length sequence, It has the capability of molecular function and DNA binding,as well as catalase and nuclease activities. It also takes part in DNA metabolic and biological processes.

6. Structural maintenance of chromosomes protein 2 (Fragment) (A0A0K1NZV4):1027 AA sequence

7. Genome polyprotein(A0A6M6R932): has a sequence of 3215 AA

Overall, 10 gamma globulins of human and 4 proteins of Indian soapberry were considered to assess the bonding and binding efficacy between the protein interactions as the software permits proteins with <1000 AA. Globulin and phytal protein of co-immuno precipitated proteins were used for developing the models with *HawkDock* software. The 1st best protein complex with highest score among the top 10 models developed during the assessment, was considered for PDB sum analysis. The results indicated the interaction levels in terms of prevailing sulphide bridges, Hydrogen bonds, non-contract bonds between the two biomolecules. The different combinations of interactions between 8 gamma globulins and 4 phytal proteins of Indian soapberry were assessed (Table :2)

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Table 2. *Interactions between the plant proteins and serum gammaglobulins of RA subjects*

S.No	Proteins involved			Bonds and bridges			
	RA Serum gamma globulin	Indian soapberry seed (Plant)	Interactive Interfaces No	salt bridges	H bonds	Non-bonded contacts	bonding score
1	**Q3KNS1**	A0A0U1XK40	18-26	2	6	157	-6051
		A0A7H1CQR4	23-20	1	4	153	-5086
		A0A0U1XGS5	15-14	1	3	104	-6041
		A0A0K1P088	20-22	3	5	129	-5794
2	**Q8TCD0**	A0A0U1XK40	15-13	3	4	95	-5057
3	**Q04695**	A0A0U1XK40	14-19	1	2	118	-5057
		A0A7H1CQR4	18-17	0	4	163	-4611
		A0A0U1XGS5	13-14	3	1	88	-4646
		A0A0K1P088	13-16	4	2	119	-4380
4	**Q6N092**	A0A0U1XK40	15-18	4	5	153	-6098
		A0A7H1CQR4	20-22	2	6	185	-6180
		A0A0U1XGS5	18-17	2	7	136	-4938
		A0A0K1P088	23-20	2	10	186	-5542
5	**A0A0X9T7T4**	A0A0U1XK40	16-14	1	4	123	-5738
		A0A7H1CQR4	17-15	1	4	105	-4834
		A0A0U1XGS5	20-13	1	4	84	-4930
		A0A0K1P088	20-19	2	5	125	-4686
6	**A0A024R6I7**	A0A0U1XK40	18-14	2	7	119	-5658
		A0A7H1CQR4	25-27	2	4	209	-6599
		A0A0U1XGS5	14-18	2	8	109	-3740
		A0A0K1P088	21-19	3	3	112	-5206
7	**HOY300**	A0A0U1XK40	18-18	1	1	127	-5286
		A0A7H1CQR4	15-17	1	5	120	-5225
		A0A0U1XGS5	19-31	2	3	200	-5063
		A0A0K1P088	19-24	2	4	208	-4881
	unique proteins						
1	B2RDW1	A0A0U1XK40	12-14	3	3	91	-5425
		A0A7H1CQR4	17-21	-	0	170	-4795
		A0A0U1XGS5	11-15	2	4	108	-4230
		A0A0K1P088	20-16	2	3	157	-4443
2	Q6DHW4	A0A0U1XK40	21-16	-	7	145	-5745
		A0A7H1CQR4	24-21	-	12	147	-6242
		A0A0U1XGS5	14-12	-	2	45	-3759
		A0A0K1P088	23-19	-	10	143	-5194
3	Q7Z351	A0A0U1XK40	12-14	3	3	91	-6479
		A0A7H1CQR4	32-25	2	5	225	-6999
		A0A0U1XGS5	16-18	-	3	144	-4598
		A0A0K1P088	12-14	2	6	116	-5723

The analyses indicated the bonding patterns while assessing their binding capabilities in different combinations between the 4 phytal proteins of Sapindus and the serum gammaglobulin proteins of RA affected subjects. Among the 40 different combinations, phytal protein (A0A0U1XK40), has shown higher bonding score while bonding with the serum globulin proteins Q3KNS1, Q8TCD0, Q04695, A0A0X9T7T4 and B2RDW1 and the scores being -6051, -5057,-5738 and,-5425 respectively (Fig5a) 10% of the unique proteins and 50% of the serm

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globulins showed affinity towards A0A0U1XK40. In contrast, serum globulin proteins such as Q6N092, A0A024R617 and 2 unique proteins (Q6DHW4, Q7Z351) showed highest bonding scores with phytal protein A0A7H1CQR4 represented by-6098,-6599, -6999 and -6242 respectively (Fig.5b). 20% of unique proteins and 20% of the globulin proteins had affinity towards AOA7H1CQR4.

protein of human and the Sapindus seed protein A0A7H1CQR4 (ATP synthasse alpha sub unit) showed the interactive regions between 32-25. 2 salt bands, 5 Hydrogen bonds and 225 (Fig.6-a,b,)

Similarly, the plant protein of *Sapindus* species ATP synthase alpha subunit showed a close interaction with another RA serum globulin protein A0A024R617. The

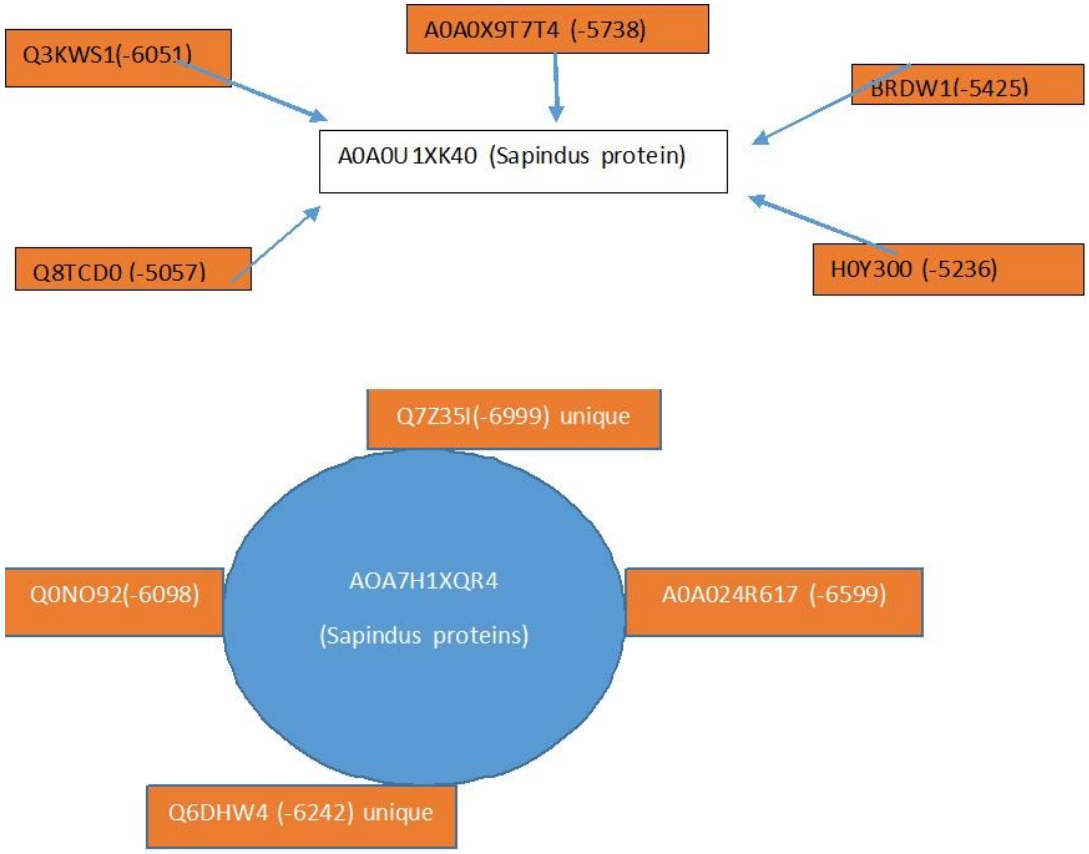
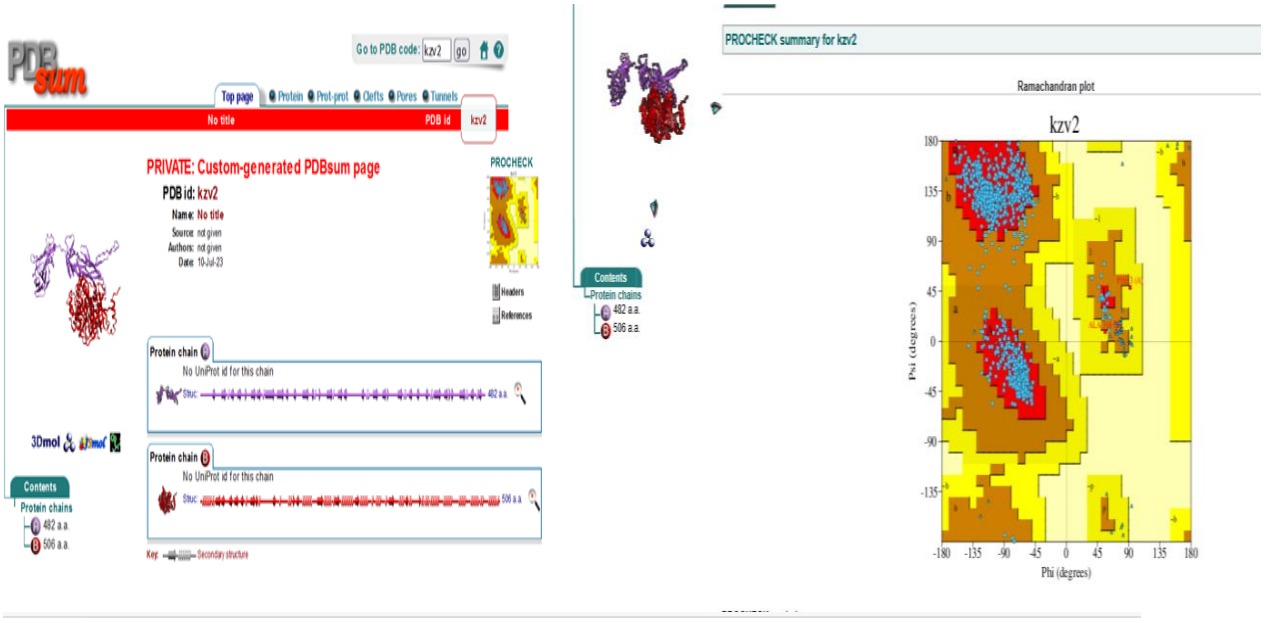


Figure 5a,b. *Interaction efficacies of phytal proteins and serum proteins of Rheumatoid arthritis subjects.*

The PPI of the proteins are often well explained through their banding patters of covalent bons, hydrogen bonds and non-bonded contact between the two proteins at certain points known as interactive interfaces. During the current study the interactive surfaces represented by each of the 4 selected phytal protein varied with that of each globulins as well as the bonding pattern. Based on their Number of Hydrogen bonds, slat bands and the other interactions the better bonding was identified in the protein-protein interaction.

The Strongest interaction was noticed between Q7Z351(Ig G like uncharacterized protein) of arthritis serum

respective 25-27 interfaces of both the proteins interacted with 2 salt bridges, 4 hydrogen bonds and 209 non bonded contacts.and the score being-6599 and bonding energy being-32.5kcal/. Another *sapindus* protein RuBISCo (A0A0U1XK40) reacts with Q3KNS1 (Patched domain - containing protein 3) which is the main protein that controls sperm development or sperm function and as well effected by Rheumatoidism of muscles. The interaction may be due to 2 salt bridges, 6Hydrogen bonds and 1578 non-bonded contacts at 18-26 interfaces of the protein and the score being-6051.



1. Ramachandran Plot statistics

		No. of residues	%-tage
Most favoured regions	[A,B,L]	786	92.4%
Additional allowed regions	[a,b,l,p]	63	7.4%
Generously allowed regions	[~a,~b,~l,~p]	2	0.2%
Disallowed regions	[XX]	0	0.0%
Non-glycine and non-proline residues		851	100.0%
End-residues (excl. Gly and Pro)		4	
Glycine residues		74	
Proline residues		59	
Total number of residues		988	

Based on an analysis of **118** structures of resolution of at least **2.0** Angstroms and *R*-factor no greater than **20.0** a good quality model would be expected to have over **90%** in the most favoured regions [A,B,L].

2. G-Factors

Parameter	Score	Average Score
Dihedral angles:-		
Phi-psi distribution	-0.14	
Chi1-chi2 distribution	0.37	
Chi1 only	0.09	
Chi3 & chi4	0.69	
Omega	-0.62*	
		-0.10
Main-chain covalent forces:-		
Main-chain bond lengths	0.58	
Main-chain bond angles	0.12	
		0.32
OVERALL AVERAGE		0.08

G-factors provide a measure of how **unusual**, or out-of-the-ordinary, a property is.

Values below -0.5* - unusual
Values below -1.0** - highly unusual

Important note: The main-chain bond-lengths and bond angles are compared with the Engh & Huber (1991) ideal values derived from small-molecule data. Therefore, structures refined using different restraints may show apparently large deviations from normality.

Figure 6a. *Interaction efficacy of phytal protein (AOAO7H1XQR4-ATP synthase subunit alpha) and serum protein (A0A024R617Alpha-1-antitrypsin)of Rheumatoid arthritis subjects.*

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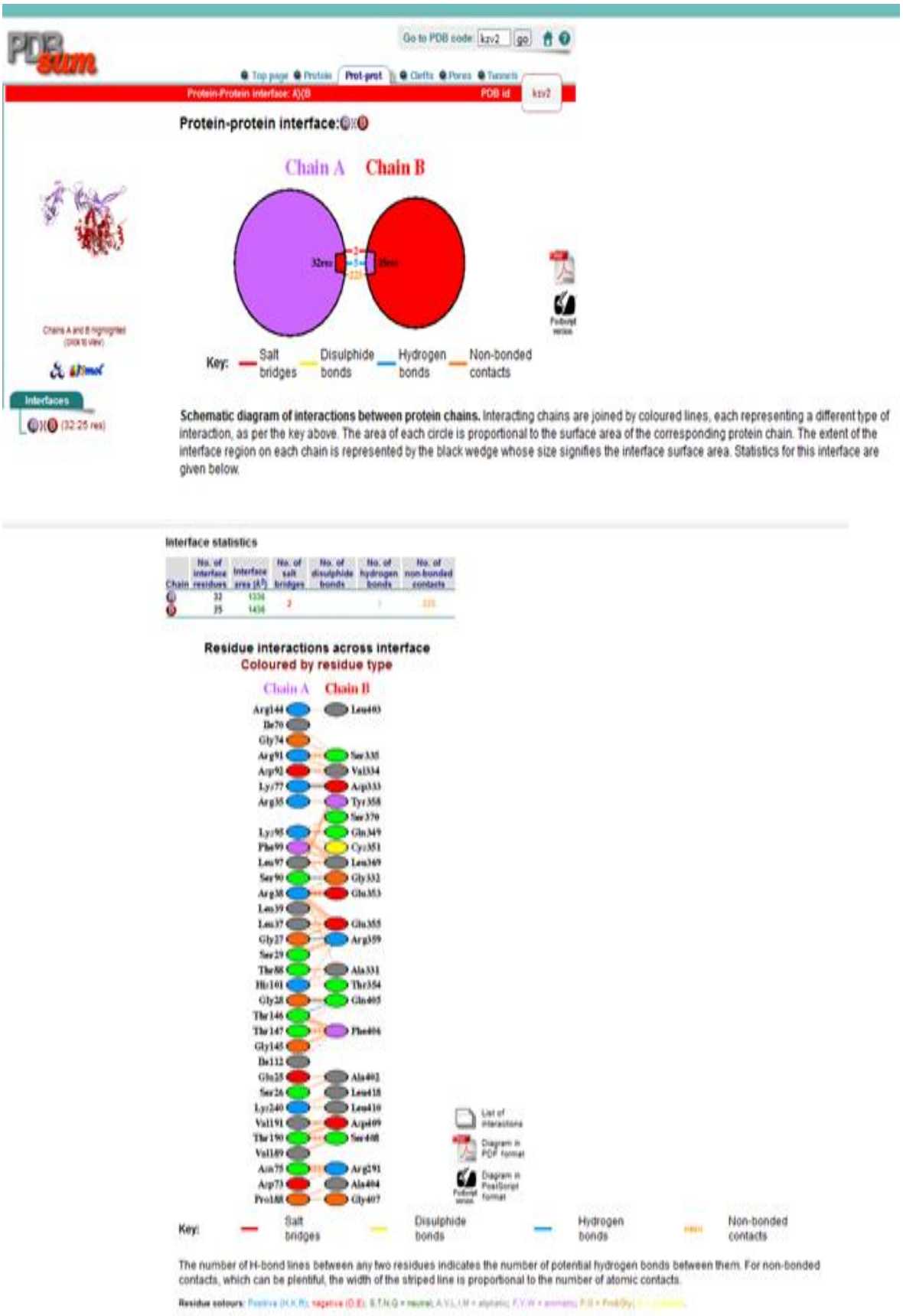

Figure 6b. *Bonding efficacies of phytal proteins(AOAO7H1XQR4-**ATP synthase subunit alpha**) and serum proteins (A0A024R617Alpha-1-antitrypsin) of Rheumatoid arthritis subjects.*

Discussion

Previous studies indicated that natural plant extracts or mixed herbal compounds effectively regulate the immune system to alleviate RA by inhibiting pro-inflammatory cytokines. (Zhao et.al.,2021). Studies were carried out to identify potential medicinal plants and natural bioactive compounds against arthritis. Most of the observations were considered for clinical and preclinical studies to control RA based on in-vitro and in-vivo studies with respect to their potential action as anti-inflammatory phytochemicals. In the Indian context, a list of 18 species of medicinal plants that can reduce the arthritis inflammation was cited due to their phytochemical constituents (Gandhi et.al., 2022) and a perspective plant-based therapy suggested based on 37 plants (Gautam et.al.2020) recently. Another perspective of plants in Rheumatoid arthritis studies is based on the nutritional intervention in the form to control rheumatoid arthritis inflammation or preventive role (Alwarith et.al., 2019). Very few studies attempted to establish a relation between the phytal proteins and the few expressed proteins in the serum of rheumatoid arthritis subjects. The study is an attempt to understand and establish a relation between the two protein counterparts and consider it for either diagnostic or control purposes. The protein-protein intereaction studies between the serum gamma globulin poroteins of rheumatoid arthritis patient and that of the different plant proteins of leaves of *Basella*(Malabar basil), *Murayya koeinig* and seed proteins of *Sapindus* (Indian soapberry) indicated development of the precipitin bond due to immunoprecipitation. The co-immunoprecipitation indicated the presence of proteins in the precipitin bonds.

30 proteins from serum gamma globulin of RA subjects (*Homo sapiens* database) and 7 of plant origin (*Sapindus* database) were identified from the protein precipitation band. Of the 30 proteins from serum, 4 were unique proteins with Ig G-like domains and without any representative genes, 2 virus-like proteins. and the 24 proteins with gene representation considered for the Metascape analysis. Studies on the rheumatoid arthritis proteins reflected different compositions in different geographical conditions viz Pakistan (Jaahangir et.al.2022) China (Hu et.al.,2022), Ireland (Ardle et.al.,2021) and the representation was 10,24 and 8 differentially expressed proteins. Of these, only HP and SERPINA1 were observed among the 30 proteins of the current study.

The Metascape analysis of human immunoglobulin proteins identified from the co-immunoprecipitation bonds showed the Reactome gene sets of neutrophil degranulation, keratinization, and formation of cornified envelopes well as GO annotations of inflammatory response. Reactome-based genes especially represented the neutrophil degranulation process during the current study Neutrophils are heroes of the immune system especially regulate innate and adaptive immune responses. They infiltrate sites of inflammation and neutrophils and aberrant neutrophil cell death results in pathogenesis of various system autoimmune diseases (Mariana J Kaplan 2013). NET formation and NETosis could be the reasons for discharge of citrullinated antigens in RA (Lingshu Zhang,2022).

The previous identified 12 biomarkers (CRP, epidermal growth factor, interleukin (IL) 6, leptin, matrix metalloproteinase 1 (MMP-1), matrix metalloproteinase 3 (MMP-3), resistin, serum amyloid A (SAA), tumor necrosis factor receptor type I (TNF-RI), vascular cell adhesion molecule 1 (VCAM-1), vascular endothelial growth factor A (VEGF-A), and cartilage glycoprotein 39 (YKL-40)) with an objective to measure the RA disease activity (Jurgens et. al., 2022). The current Phytal protein and serum gamma globulin protein interactions are unique studies. Based on the co-immune precipitation between the two protein complexes, protein-protein interactions from the precipiting band were studied. The identified proteins were further characterized and bonding pattern was checked with *HawkDock*, MMGB and PDBsum. The phytal proteins of the predominance were RuBisco and ATPsynthase alpha subunit. The results highlighted the interactive conditions of specific seed proteins of Indian soap nut(A0A0U1XK40) Ribulose bisphosphate carboxylase large chain, and ATP synthase alpha subumit (A0A7H1CQR4) with that of three unique proteins (B2RDW1, Q6DHW4, and Q7Z35) of considerable interest. Sapindus protein RuBISCo (A0A0U1XK40) reacts with Q3KNS1 (Patched domain – containing protein 3) which is the main protein that controls sperm development or sperm function and as well effected by Rheumatoidism of muscles. The interaction may be due to 2 salt bridges, 6Hydrogen bonds and 1578 non-bonded contacts at 18-26 interfaces of the protein and the score being-6051. ATP synthase alpha subumit indicated bonding with uncharacterized protein with Ig G like domains (Q7Z351) and, A0A024R617 (Serpin peptidase inhibitor, clade A (Alpha-1 antiproteinase, antitrypsin). The strong bonding patterns and low energy levels indicate that they can be used for identifying the Q3KNS1 and, unique protein Q7Z351 and A0A024617 respectively which can trigger auto immune disorders and their consideration as biomarkers.

Acknowledgments

We thank the Management of SVKM for providing good laboratory conditions, Dr.Shah Pathology labs for the Rhematoid arthritis subject samples, IITB for the protein analysis and Prof.Krutika Desai, Principle, Mithibai College for support and encouragement.

Subject of interest: Nil

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