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PARTIAL CHARACTERIZATION, CYTOTOXIC AND GENOTOXIC PROPERTIES OF GLYCOPROTEINS FROM SYZYGIUM CUMINI LAM. SKEELS (BLACK PLUM)

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Keywords:

Black jamun, Trichloroacetic acid, Human peripheral blood mononuclear cells, Hemagglutination, Hemagglutination inhibition, Genotoxicity

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ABSTRACT: In nature, lectins or glycoproteins are ubiquitous, abundantly distributed. Lectinsplayan an important role in plant defense mechanism against microorganisms. This fact has greatly attracted many research groups working in the field of biotechnology, agriculture, and medicine. Jamun or black plum is a fruit with many health benefits. This paper focuses on the phytochemical investigation of trichloroacetic acid precipitated proteins from leaves, pulp, and seeds. The glycoproteins were characterized by hemagglutination, hemagglutination inhibition, and tested the stability at different temperatures and pH on blood groups by and tested their efficacy on human Peripheral Blood Mononuclear Cells. Phytochemical investigations of trichloroacetic acid precipitated test sample defined the occurrence of protein and absence of other phytochemicals. Blood group O^+ showed a quick response of agglutination. Both pulp and leaf extracts showed cytotoxicity and genotoxicity on human Peripheral Blood Mononuclear Cells in a dose-dependent manner. Glycoproteins were stable at varying temperatures and pH. The current study results indicated that the jamun fruit extract is also a potent anti-proliferative agent against lymphocytes. However, further tests have to be done to investigate the appropriate dose, toxicity, and mechanism of anti-proliferative action of jamun pulp on human Peripheral Blood Mononuclear Cells.

INTRODUCTION: Medicinal plants are very important for human health. According to the World Health Organization (WHO), more than 80% of the population in the world depends upon traditional medicine for their primary healthcare [1]. Medicinal plants contain diverse bioactive substances, which act against various diseases.

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Plant proteins, phytochemicals, and their derivatives from the traditional plants are attracting great interest on account of their various biological activities, such as cell agglutination, antiviral, anti-fungal and anti-neoplastic activities, and ably bind to carbohydrates reversibly and agglutinate the cells [2, 3].

Plant glycoproteins are the key classes of pharmaceuticals used to treat various medical conditions. One of the plant protein sislectin, which is glycoprotein. The majority of plant lectins are fascinated targets of researchers in many areas of medical research, including aging, cancer treatment, and other clinical problems as they are

cell agglutinating proteins that bind reversibly to mono-and oligosaccharides with high specificities. As plant lectins resist digestion, the intact lectins can enter circulation, and once internalize into cells; they involve in various activities. One among those is induction of apoptosis or cell cycle arrest.

In the present health scenario, malignancies are increasingly important causes of morbidity and mortality and also considered a major life-threatening next to cardiovascular diseases [4, 5]. The lymphatic system transports lymph that contains white blood cells known as lymphocytes. The main role of lymphocytes is to fight disease in the body and play an important role in the immune system. Human Peripheral Blood Mononuclear Cells (hPBMCs)/ lymphocytes are responsible for adaptive immunity. These are continuously circulating along with blood and lymph and are useful in research fields like immunology, vaccine development, hematological malignancies and lymphoblastic leukemia are caused by a group of genetic alterations that result in the uncontrolled proliferation of lymphoid progenitor cells [6]. Like any other cancer, lymphatic system is also susceptible to cancer, which is termed lymphoma. This affects the white blood cells of lymphatic system. Lymphoma treatment involves radiotherapy, stem cell transplantation, steroid treatment, and surgery. Though many treatment patterns are available, there is still a reasonable percentage of patients who are not achieving complete cure or reverting later than dying of disease.

Syzygium cumini (*S. cumini*) (L.) Skeels (jambolan, jamun) or *Euginea jambolana* (L.) Skeels belongs to family Myrtaceae. This plant is a rich source of alkaloids, jambosine, glycoside jambolin, anthocyanins, vitamins, minerals, proteins and antimellin which can be used to treat various diseases. In Ayurveda, jamun plant plays a major role in the treatment of dental problems, liver diseases, cancer, digestive disorders, skin diseases, diabetes, throat, spleen diseases, ringworm infections, *etc.* A previous report showed that jamun possesses cancer defensive properties in DMBA-induced (N, N-Dimethylbenzylamine) Swiss albino mice [7]. Reports say that at higher concentrations, pulp has capability to slow down the proliferation of cancer cell lines, MCF-7, HeLa, HEPG2, HL 60, and U2518. In the present study, we compared the phytochemicals of dried leaf powder, lyophilized seed and pulp samples with Tri Chloro Acetic acid (TCA) precipitated plant material. We made an attempt on the utility of TCA precipitated leaf, pulp and seed proteins by hemagglutination activity (HA), hemagglutination inhibition (HAI), anti-proliferative and DNA fragmentation on hPBMCs.

MATERIALS AND METHODS: *Eugenia jambolana* blooms in March and yields fruitsin May/ June. Since the fruit is seasonal, leaves and fruits were collected during the same season from Kolar area. The collected materials were authenticated by Dr. Madhava Chetty, Assistant Professor, Sri Venkateswara University, Tirupati and the voucher specimen number given was 3017. After washing the fruits with distilled water, pulp portion was removed with a clean knife and spatula, and seeds were separated. Crushed seed and pulp were lyophilized at the Indian Institute of Sciences, Bangalore, and stored at -20 ºC till further analysis. The collected leaves were washed, air dried, and made into fine powder, and storedin amber coloured bottles at room temperature.

Protein Extraction: To 4 gm of each sample, 200 mL of petroleum ether was added and incubated for 1-3 h at room temperature on a magnetic stirrer. The sample was clarified at maximum speed for 15 min at room temperature. The macerated pellet (with 100 mL of phosphate buffer saline (PBS pH 7) solution) was centrifuged for 45 min at 3000 rpm. To precipitate the proteins, 50-100 mL of 10% (TCA) was added to the clarified solution [9]. It was left at room temperature for 30 min, centrifuged for 30 min at 3000 rpm. Pellet was washed two times with distilled water to remove excess acid. To the pellet, 500 µl of PBS/water was added and stored at 4 ºC. Protein concentration from fruit, leaf, and seed extracts was quantified in spectrophotometer by Bradford's reagent by using BSA as standard [10].

Phytochemical Analysis: About 1mg of TCA precipitated proteins from leaf, seed and pulp were dissolved in distilled water was used for the analysis of phytochemicals *viz.*, fixed oils, glycosides, carbohydrates, phenolics, flavonoids, proteins, saponins, gums/mucilage, and resins by following the standard methods [11].

Hemagglutination Activity (HA): Agglutinating property plays a critical role in early investigations of ABO blood group system. After obtaining institutional ethics clearance (SDUAHER/Res. Project/ 115 /2015-16), A^+, B^+, O^+ and AB^+ group blood from healthy human volunteers were collected in Ethylenediaminetetraacetic acid (EDTA) vacutainers, centrifuged and discarded the plasma and washed two times in PBS. Finally, 1% of erythrocyte solution was prepared in a ratio of 1:9 (blood:PBS) and stored at 4 ºC for the hemagglutination and hemagglutination inhibition assays11,12. The three concentrations of all the samples used were 45 µg/mL, 90, and 180 µg/mL. The sample and 1% erythrocyte were mixed in 1:1 ratio in a 12 well glass plate, left undisturbed at room temperature, and monitored for hemagglutination. The activity of hemagglutination is expressed as a hemagglutinating unit (HU). Hemagglutination time was noted down for all the samples. Controls were maintained as RBCs, PBS+RBCs, and RBCs + distilled water.

Hemagglutination Inhibition Assay (HAI): Hemagglutination inhibition assay was carried out in a 96 well plate [11, 12]. Working concentrations of all the sugars ranged from 0.5 mM – 2.5 mM with 5 mM variation were made. The protein concentration taken was 180 µg/mL and was mixed with equal volumes of all the sugars in a 96 well plate and incubated for one hour at room temperature. After one hour, eight volumes of 1% RBC (O+) were added to all the wells, and the time taken for inhibition was noted down.

Effect of Temperature and pH: To find out the thermal stability, all the seed, pulp and leaf proteins were exposed to a range of temperatures from 30 ºC – 100 ºC [2]. About 1 mg/mL concentrated proteins were kept at respective temperatures for 30 min and then brought to room temperature. Equal volumes of 1% erythrocyteswere added and observed for HA. Buffers of pH, 2 (Glycine-Hcl); 4 (Sodium acetate); [1]3 (Sodium phosphate); 9 (Tris-Hcl); [11] (Glycine-NaOH) and [14] (NaOH) were prepared to test the effect of pH on the proteins. Equal volumes of proteins (1 mg/mL) and buffers of respective pH were mixed and incubated at 25 ºC for 5 h. After incubation, samples were brought back to neutral pH and proceeded for HA with O+ blood group.

Collection of Peripheral Blood and Isolation of Lymphocytes: The leftover O^+ blood was collected from the central diagnostic laboratory of Sri Devaraj Urs medical college. To 1 ml of the blood, added an equal volume of cold PBS (pH 7), mixed the contents properly and carefully layered onto 2 mL Ficoll in a falcon tube without getting mixed up with Ficoll, and spun down the contents at 3000 rpm for 30-45 min at room temperature [14]. During the centrifugation process, the PBMCs moved from the plasma and were suspended in the density gradient, separating them from erythrocytes and granulocytes. Removed plasma down to about 1 cm above the buffy coat, discarded, then aspirated the buffy coat (the white layer lying on top of the red cells, it should come as one layer). The PBMC/buffy coat layer was washed two times in PBS. The PBS as the supernatant was then removed and discarded.

Roswell Park Memorial Institute (RPMI) medium was prepared by mixing 40 mL of RPMI, 10 mL of fetal bovine serum (FBS) and 200 µL Antibiotic / antimycotic (Antibiotic antimycotic solution with Streptomycin 10 mg/20 mL, 10,000 U Penicillin, Amphotericin B and 0.9% normal saline). About 1 mL of RPMI medium was dispensed into falcon tubes, added 30 µL of Phytohemagglutinin (PHA), 100 µL of hPBMCs, and incubated with an atmosphere of 95% air and 5% CO_2 at 37 ºC for 4 h. Before incubation, counting the number of cells from 10 µL [15, 16].

After incubation, different concentrations of pulp and leaf proteins (50 µg, 100 µg, 150 µg, and 200 µg) were added to the respectively labeled tubes. Controls were DMSO and hPBMCs without protein. Tubes were incubated as above mentioned conditions and clarified at 3000 rpm for 45 min. The supernatant was discarded, and cells in approximately 200 µl were stored at -20 ºC for DNA fragmentation assay.

Trypan Blue Dye Exclusion Method: About 10 µl of cells with 10 µl of Trypan blue (0.4% Trypan blue in a balanced salt solution) and cell suspension were mixed (1:1) and left for 10 min at room temperature. Transferred 10 µl of this onto hemocytometer, and observed under a microscope for viability count [16]. Percent viability was calculated by applying the following formula.

Percent viability = Number of viable cells / Total number of cells × 100

DNA Fragmentation Assay: About 200 µl (approximately 1,260 cells in pulp and 980 cells in leaf per 200 µl) of the samples were taken further for DNA fragmentation assay. For lysis buffer, solutions, *viz.* 1M Tris, 20mM EDTA, and 1%SDS were prepared as per the protocol mentioned by Richard Patten [17]. An aliquot of 200 µl lymphocytes was centrifuged at 2000 rpm for 5 min, and the supernatant was discarded. To the pellet, 20 µl of TES buffer was added and mixed properly to lyse the cells. To this, 10 µl of RNase was added and incubated for 1 h at room temperature and incubated further for 1 h 30 min at 50 ºC with proteinase K. Total sample was loaded in 1% agarose gel and ran the gel at 80-100 volts. After the run the gel image was taken by using UV-trans illuminator, and the image was documented.

Statistical Analysis: All the experiments were conducted in triplicates, and standard errors were calculated wherever needed.

RESULTS: Plant, animal or microbial originated glycoproteins are non-immune and have specificity to carbohydrate moieties. Shrikant *et al.,* [18] concluded that Eugenia uniflora seed extracts have potential antibacterial properties. This made us validate the difference among the three parts of the plant, *i.e.*, leaf, pulp, and seed extracts on PBMCs, so that these can be used as anti-proliferative agents. In the present study, we used the TCA precipitated proteins isolated from leaves, pulp, and seed and the specific concentrations of the proteins were checked by Bradford's assay.

Phytochemical Analysis: TCA precipitated proteins from leaf, seed and pulp were analyzed for phytochemicals *viz.*, fixed oils, glycosides, carbohydrates, phenolics, flavonoids, proteins, saponins, gums/mucilage, and resins. The analysis showed negative results for all the phytochemicals except protein. Phytochemical tests for protein indicated the presence of proteins but not any other phytochemical.

HA Assay: Our aim is to know whether the TCA precipitated proteins of Jamun leaf, pulp, and seed have any agglutination property with human red blood cells. Their hemagglutination capacity assayed different concentrations of Eugenia jambolana leaf, pulp, and seed protein activity with human O+, A+, B+, and AB+ blood groups. All the blood groups showed dose-dependent agglutination at 45 µg/mL, 90, and 180 µg/mL concentrations. Seed extract with A^+, B^+, and AB^+ blood groups took more than 30 min to agglutinate when compared to leaf, pulp, and O^+. Among the groups, the O^+ blood group showed agglutination more rapidly than the other three groups **Fig. 1**. This is in agreement with the findings of Oliveira *et al.*, where the seed extract was used [19].

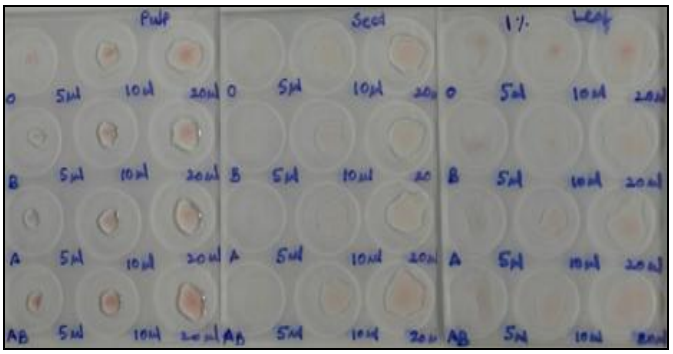

FIG. 1: TEST FOR HEMAGGLUTINATION OF INC-REASING CONCENTRATIONS OF PULP, SEED AND LEAF TRICHLOROACETIC ACID PRECIPITATED PROTEINS

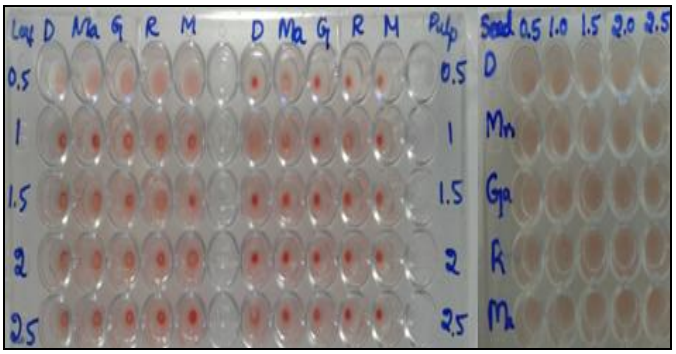

FIG. 2: TEST FOR HEMAGGLUTINATION INHIBITION ASSAY WITH INCREASING CONCENTRATIONS OF SUGARS ON TRICHLOROACETIC ACID PRECIPITATED PROTEINS OF LEAF, PULP AND SEED

HAI Assay: Carbohydrate binding specificity of leaf and pulp proteins was compared among dextrose, mannose, galactose, ribose, and maltose sugars at varying concentrations. Leaf proteins, when mixed with 0.5 mM concentration of all sugars, showed agglutination within 25 min, whereas 2.5 mM D-mannose sugar did not show any agglutination even after one hour. In the case of pulp proteins, with glucose and D-mannose, HAI was observed with all the concentrations even after one hour.

HAI was noted with increasing concentrations of rhamnose, maltose, and dextrose **Fig. 2**. These results showed that the glycoproteins obtained from the selected plant parts are specific to mannose sugar as reported earlier in *Trigonella foenum-graecum* [8].

Effect of Temperature and pH: All three proteins were tested for their ability to withstand high temperatures and varying pH. Till 80 ºC the pulp protein was stable and showed agglutination. Thereafter, no agglutination was observed, indicative of the pulp proteins were unstable at 90 ºC and 100 ºC. On the contrary to Oliveria *et al.*, 2008, seed protein was stable only at 30 ºC [19]. Increased temperatures denatured the seed proteins, which significances no agglutination. Leaf proteins were stable till 90 ºC and agglutinated RBCs. Agglutination was not observed at 100 ºC, which indicated that leaf proteins are unstable at 100 ºC.

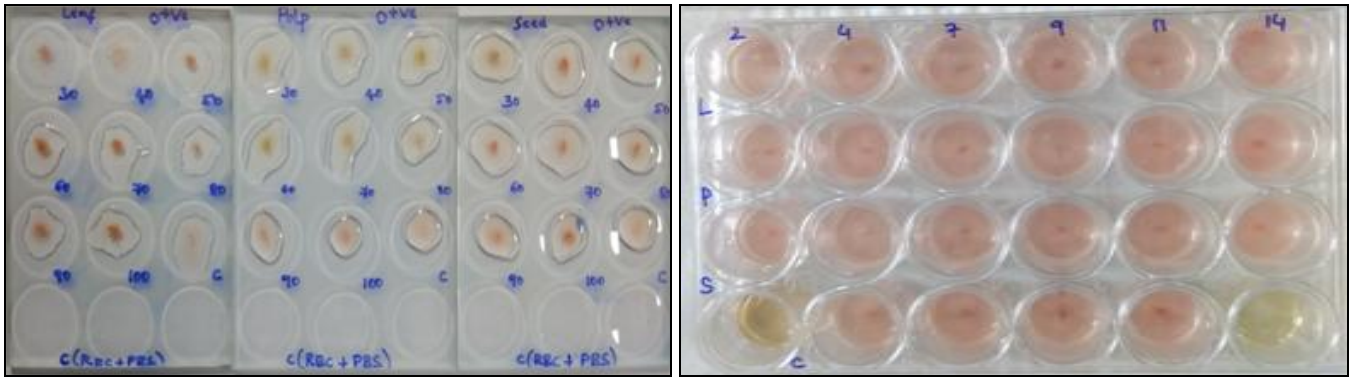

FIG. 3: TEST FOR EFFECT OF DIFFERENT TEMPERATURES (30-100) AND pH (2-14) ON TRICHLOROACETIC ACID PRECIPITATED PROTEINS OF LEAF, PULP AND SEED

Effect of Protein on Cytotoxicity of HPBMCs: Based on the results of hemagglutination assay, pulp and leaf proteins were used to treat hPBMCs from O^+ blood group and were treated with different concentration of leaf and pulp proteins (50 mg/mL, 100, 150, and 200 mg/mL), the controls were only hPBMCs and hPBMCs with DMSO.

After 72 h of incubation, cell viability count was carried out for both test samples and controls by using Trypan blue dye exclusion technique **Fig. 4**. A gradual rise in the number of dead cells/percent of non-viable cells was noted with increasing concentrations of leaf protein. Not noted a remarkable cytotoxic effect between 150 µg (dead cell 97.1%) and 200 µg (dead cell 96.7%) leaf proteins on hPBMCs. In control (wherein only hPBMCs) the percent viable cells and percent non-viable were noted as 65.3 and 34.6%, and with DMSO dead cell percentage (68.5) viable cell percentage (31.4) were noted. The effect of leaf proteins on the viability of hPBMCs was dose-dependent.

In control (only hPBMCs), the percent viable and non-viable cells were noted as 74.6 and 25.3%, and with DMSO, dead cell (57.3) and viable cell percentages (42.6) were noted in **Table 1**. It was noted that leaf proteins are comparatively more cellular toxic than pulp proteins **Fig. 5** and **6**.

TABLE 1: PERCENT VIABLE AND NON-VIABLE CELLS WHEN HPBMCS WERE TREATED WITH DIFFERENT CONCENTRATIONS OF LEAF AND PULP PROTEIN

Treatment	Percent of viable cells		Percent non-viable cells	
	Pulp	Leaf	Pulp	Leaf
Control 1	74.6 ± 0.58	65.3 ± 1.15	25.3 ± 0.58	34.6 ± 1.15
Control 2	42.6 ± 1.15	31.4 ± 1.73	57.3 ± 1.13	68.5 ± 2.31
Treatment 1	19.3 ± 0.58	5.5 ± 0.57	80.6 ± 1.15	94.4 ± 0.57
Treatment 2	19.1 ± 1.15	3.5 ± 0	80.8 ± 2.30	96.4 ± 0
Treatment 3	12.2 ± 0.58	2.8 ± 0.57	87.7 ± 4.62	97.1 ± 0.57
Treatment	449.6 ± 1.1	53.2 ± 0.57	90.3 ± 1.15	96.7 ± 0.57

Control 1: Only HPBMCS; control 2: HPBMCS+ DMSO; treatment 1: HPBMCS+50 µG/ML pulp/leaf protein; treatment 2: HPBMCS+100 µG/ML pulp/leaf protein; treatment 3: HPBMCS+150 µG/ML pulp/leaf protein; treatment 4: HPBMCS+200 µG/ML pulp/leaf protein. Values are expressed as Mean ± S.E.

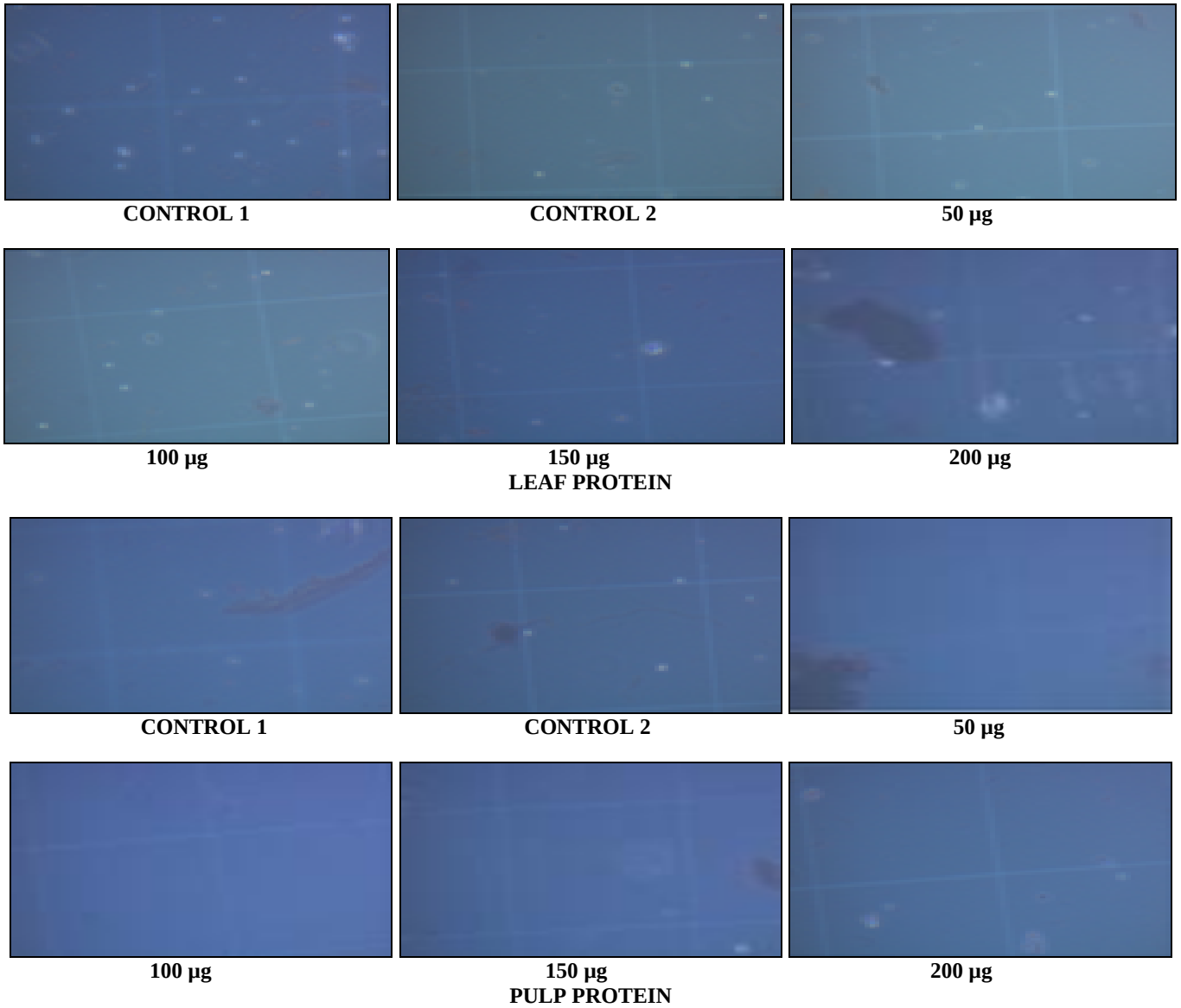

FIG. 4: IMAGES OF CELLS IN CONTROLS AND TREATMENTS WITH LEAF AND PULP PROTEINS AFTER TRYPAN BLUE DYE EXCLUSION. HPBMCS WERE TREATED AND AFTER INCUBATION, CELLS WERE STAINED WITH TRYPAN BLUE, VISUALIZED AND CAPTURED UNDER MICROSCOPE

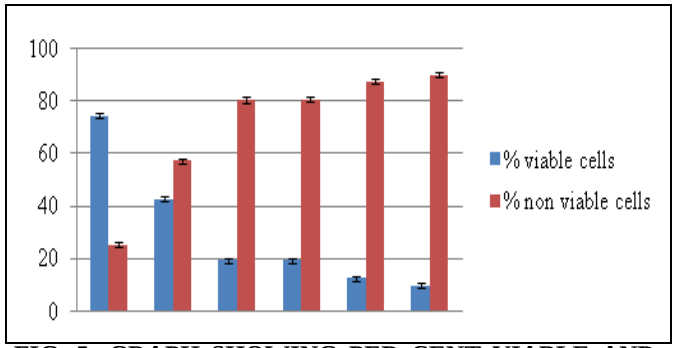

FIG. 5: GRAPH SHOWING PER CENT VIABLE AND NON-VIABLE CELLS WHEN PBMCS WERE TREATED WITH DIFFERENT CONCENTRATIONS OF PULP PROTEIN. VALUES ARE EXPRESSED AS MEAN ± S.E. bars from left to right: 1: PBMCS only; 2: PBMCS + DMSO; 3: PBMCS + 50 µg/ml pulp protein; 4: PBMCS + 100 µg/ml pulp protein; 5: PBMCS + 150 µg/ml pulp protein; 6: PBMCS + 200 µg/ml pulp protein

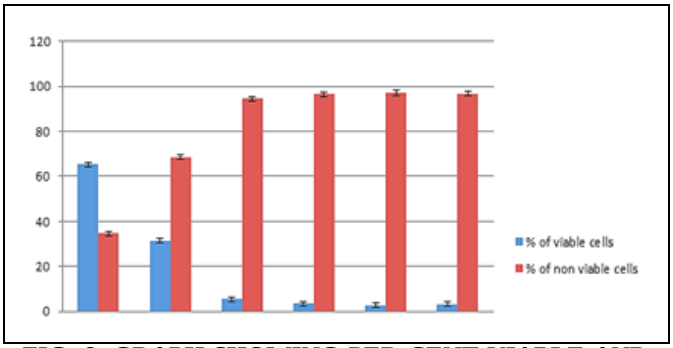

FIG. 6: GRAPH SHOWING PER CENT VIABLE AND NON-VIABLE CELLS WHEN PBMCS WERE TREATED WITH DIFFERENT CONCENTRATIONS OF PULP PROTEIN. VALUES ARE EXPRESSED AS MEAN ± S.E. bars from left to right: 1: PBMCS only; 2: PBMCS + DMSO; 3: PBMCS + 50 µg/ml leaf protein; 4: PBMCS + 100 µg/ml leaf protein; 5: PBMCS + 150 µg/ml leaf protein; 6: PBMCS + 200 µg/ml leaf protein

DNA Fragmentation Assay: We examined the effect of leaf and pulp proteins on DNA fragmentation of hPBMCs. The induction of DNA fragmentation is a predictive marker for DNA damage in hPBMCs. Present results showed that proteins of both leaf and pulp showed apoptotic fragmentation of lymphocyte DNA **Fig. 7**.

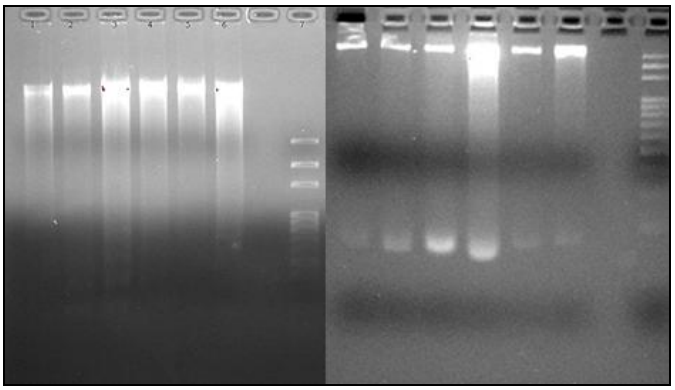

FIG. 7: AGAROSE GEL ELECTROPHORESIS OF PBMC DNA TREATED WITH DIFFERENT CONCENTRATIONS OF LEAF (LEFT) AND (RIGHT) PULP PROTEIN. Lane 1: control 1 (only PBMC), lane 2: control 2 (PBMC+DMSO), lane 3-6: PBMCS were treated with different concentrations of leaf and pulp protein. lane 3: 50 µg/ml, lane 4: 100 µg/ml, lane 5: 150 µg/ml, lane 6: 200 µg/ml and lane 7: DNA ladder (100 bp). DNA fragmentation was viewed after staining the gel with ETBR.

DISCUSSION: Results of many investigations are presenting the utility of natural products as anticancer, antioxidant, antidiabetic agents. Jamun, (Syzygium cuminii/Eugenia jambolana) is known for its antidiabetic property. Jamun as a whole (all parts), showed a reduction in the incidence of tumors and tumor burden, as the plant is a rich source of flavonoids, gallic acid, myricetin, quercetin [18]. Eugenia jambolana Lam (black plum) is effective in treating diabetes mellitus, ulcers, diarrhea, inflammation and has antineoplastic, chemopreventive properties [2].

Distinct from other blood types, people with type O^+ blood group efficiently digest and metabolize as they have high stomach acid content. The consumption of high-quality proteins safeguards the smooth functioning of the body with no metabolic stress. According to D'Adamo [20], green, red, or dark purple plums are alkaline in nature and good for type O^+ people to neutralize the digestive tract. In our present study, the proteins agglutinated O^+ blood group quickly, which indicates that when plum glycoproteins are rich in diet, they quickly agglutinate the lymphomas. Previous research showed that jamun fruit crude extract exhibited cytotoxic activity against MCF 7, Hela Cervic carcinoma cell lines, Hep G2 liver carcinoma cell lines, H 460 lung carcinoma cell lines, and U251 brain carcinoma cell lines [21, 22], but not on hPBMCs. Waqas Ahmad *et al.*, [23] used Jamun leaf powder to study the cytotoxic effects on Vero cell lines with a maximum concentration of 100 µg/mL, and almost agreement percent cell survival was noted in our study also [24].

It is clearly evident that Jamun has cancer chemopreventive properties 7 when tested on DMBA-induced (N, N-Dimethyl-bezylamine) Swiss albino mice. Genotoxic chemical exposure leads to mutations and cancer. Aqueous and ethanolic Jamun seed extracts reduced the in vitro hydroxyl radical-induced pBR322 DNA strand breaks and aqueous extract was also effective in decreasing the urethane and DMBA-induced chromosomal aberration in mice. Jagetia and Baliga [24] assessed the effect of Jamun extract on hPBMCs and observed the radiation-induced DNA damage preventive action by micronuclei assay [24, 25].

Therefore, these observations and our results clearly indicate that the Jamun extracts prevent mutagenesis and initiation of carcinogenesis. In both the extracts, concentration-dependent results have been noted in the agreement with previous studies. We showed the jamun fruit extract is also a potent anti-proliferative against lymphocytes. Jamun is one of the commonest fruits in India with high consumption rate due to low cost, safe to use and abundance.

The major advantage is its acceptability as a drug over synthetic drugs. An initial study was made to isolate a novel glycoprotein from *Eugenia jambolana* Lam leaf, pulp and seed. Both pulp and leaf extracts showed cytotoxicity and genotoxicity on hPBMCs in a dose dependent fashion. The present investigation showed the jamun fruit extract is also a potent anti-proliferative agent against lymphocytes. However, further experiments have to be done to investigate the appropriate dose, toxicity and mechanism of anti-proliferative action of jamun pulp on hPBMCs.

CONCLUSION: The current study results indicated that the Jamun fruit extract is also a potent anti-proliferative agent against lymphocytes.

However, further tests have to be done to investigate the appropriate dose, toxicity, and mechanism of anti-proliferative action of Jamun pulp on Peripheral Blood Mononuclear Cells.

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CONFLICTS OF INTEREST: Nil

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