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Bio-assay guided toxicopharmacological studies on effect *of Punica granatum* peel aqueous extract (PGPAE): insights into the phytochemical, antioxidant, antimicrobial and cyto-genotoxic manifestations

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Abstract

Punica granatum L., a deciduous fruit-bearing and decorative shrub or small tree of Punicaceae family, is widely cultivated throughout India. In folk medicine, tea decoction per say produced from fruit peels could cure a variety of disorders, such as laryngotracheal infections, inflammatory diseases, and infectious diseases. *Punica granatum* peel aqueous extract (PGPAE) was analysed for the presence of secondary metabolites like flavonoids, condensed tannins and polyphenols, *in vitro* antioxidant activities like1,1-Diphenyl-2-picrylhydrazyl (DPPH) and Ferric Ion reducing Power (FRAP) reducing activity, Fourier transformation Infrared (FT-IR) analysis, H_2O_2 induced anti-haemolysis of chicken and human RBC and anti-tissue damaging activity in chicken lung, liver and heart tissues *in vitro*. In addition PGPAE was also subjected to antimicrobial activity (Agar well diffusion method) and cytogenotoxic assays after seed priming with serially diluted concentrations (PGPAE, 10, 7.5, 5, 2.5, 1.25, 0.625 g/100 ml water) in germinating *Lathyrus sativus* L., chromosomal aberration and nucleolar degradation encompassing endpoint cytotoxic responses. PGPAE strongly inhibited H_2O_2 induced Reactive Oxygen Species (ROS)-mediated haemolysis and exhibited strong anti-tissue damaging activity. Additionally, using the *Lathyrus sativus* L. germinating root tip test this work assessed the cytotoxic and genotoxic capabilities of an aqueous extract derived from the peels of *P. granatum*. The extract pre-treatment at 0.625 g/100 ml failed to have cytostatic effects without any altered phase index (PI) than of negative control. The extract exhibited cytotoxic effects at the higher concentrations (10, 7.5, 5, 2.5, 1.25 g/ml), as well as significant anti-proliferative activity, genotoxicity, statistically significant changes in phase indices, and destruction of nucleolar morphology, which set the hallmark of apoptosis in germinating root tip cells. The extract has strong anti-microbial activity against microbial genera viz, *Escherichia coli*, *Bacillus* sp., *Klebsiella* sp., and *Priestia* sp. There were significant DPPH and FRAP reduction activity of PGPAE with 10.23±1.2 mg gallic acid equivalent (GAE)/g dry weight of total phenolics, 7.15±0.84 mg GAE/g dry weight of total flavonoids and 3.22±0.54 Catechin Equivalent /g dry weight of condensed tannin were present. Attempts to recognise and isolate the potent dietary antioxidants can be employed to mitigate oxidative stress during pathophysiological manifestations. Additionally analysis to look into the cytotoxic potential and mitostatic cum anti-proliferative actions of pomegranate epicarp to treat microbial contamination and diseases should be attempted. Dietary consumption of aqueous decoction of pomegranate peel has been proved being safe for both in lower vertebrates (chicken) and human subjects and can be effectively consumed during ailments to protect haematological health as potent cyto-protectant against ROS outbursts.

Key words: antimicrobial, anti-ROS, anti-tissue and anti-haemolytic activity, pomegranate peel aqueous extract (PGPAE), apoptosis, and nucleolar degradation.

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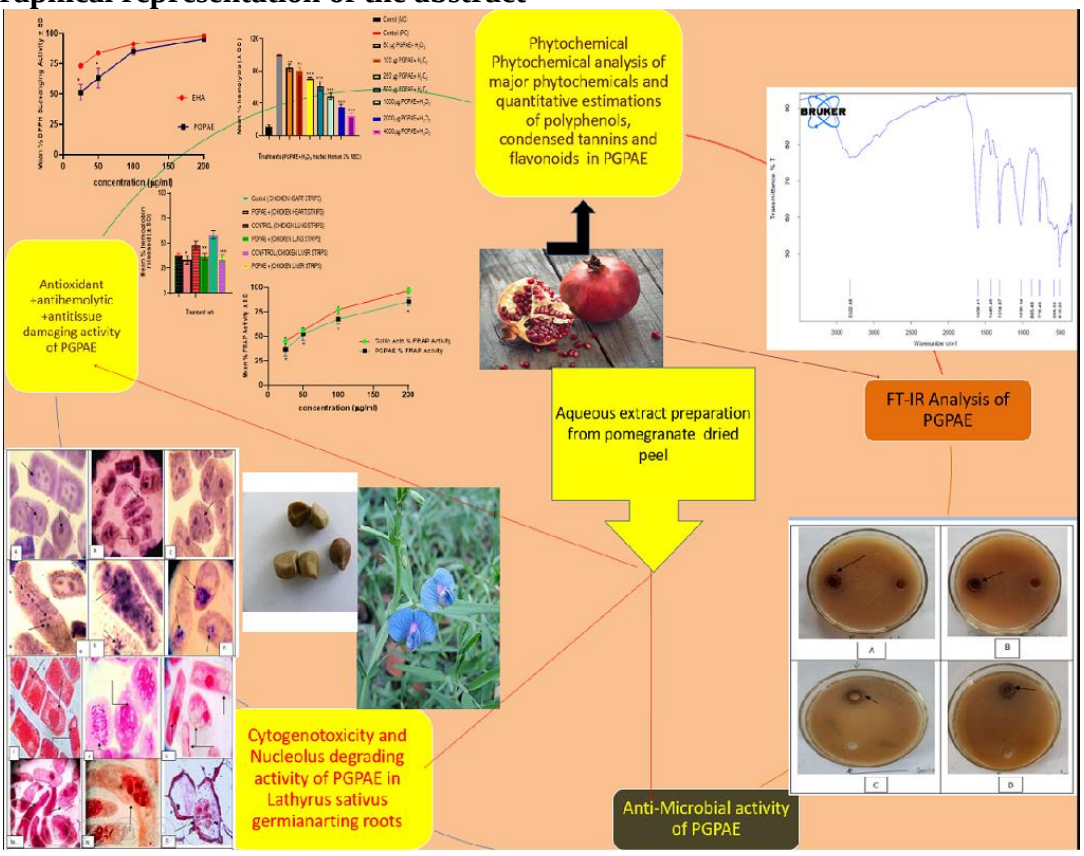
Graphical representation of the abstract



1. Introduction

For generations, pomegranate fruit has been utilized as food and as a source of health tonic. According to new molecular research, there are two species under the genus Punica, *P. granatum* L. and *P. protopunica* Balf, which can be categorized as belonging to the Punicaceae family [1]. According to Eber's papyrus (1550 BCE), Pomegranate root extract was used in ancient Egypt to get rid of tape worms. In particular, pomegranate (*Punica granatum*, family Punicaceae), has gained its popularity as a stand-by and easy-to-consume fruit, which is a storehouse of rich phytonutrients, exclusively loaded with antioxidant phytochemicals bundled with myriad health benefits. Interestingly, during the last few years among health conscious people, along with raw intake, Pomegranate juice (PJ) has been gaining popularity worldwide for its uniqueness, exclusive colour and taste, and associated health benefits [2]. Recent scientific research has established that pomegranate fruit, juice, seeds, peel, bark, flowers, leaves, and by-products of pomegranate peel extract (PPE) are rich in phytochemicals like flavonoids, phenolic acids, and hydrolysable tannins (HTs), containing antimicrobial, antioxidant, and blood pressure-lowering, as well as anti-inflammatory, anticancer, and antiulcer properties [3, 4]. The antibacterial activity of plant extracts is considered to be the basis for practical applications in food industries, especially in the preservation of both raw and processed food, medications, complementary and alternative medicine, food packaging, textiles, cosmetics and natural therapies [5, 6, 7]. Micro-organisms are becoming increasingly resistant to synthetic medicines and antibiotics as a result of antibiotic misuse. Thus, the extraction of anti-infective chemicals from plants and animals is currently the focus of research [8]. Epi-, Meso- and Endocarp of different seasonal fruits are rich in different secondary metabolites, polysaccharides, and bioactive enzymes. It is already established that the aqueous *Punica granatum* leaf extract exhibits antioxidant potential [9]. Recently polyphenol oxidase in addition to peroxidase activity was recorded in different apple cultivars and banana cultivars for their functional importance related to fruit health and storage [6, 10]. Purification of polyphenol oxidase and precipitation by $(NH_4)_2SO_4$ had also been characterized from Artichoke (*Cynara scolymus* L.) [11]. Peroxidase and polyphenol oxidase enzyme

activity with the changes of pH as a factor had also been recorded from Kalipatti sapota (*Manilkara zapota*) [12]. Tao *et al.* (2013) [13] reported recently, the purification and characterisation of elevated polyphenol oxidase activity with time variation from Jackfruit (*Artocarpus heterophyllus*) bulbs. These findings established that fruit peels rich in polyphenol oxidase and peroxidases, help to combat phyto-parasites during fruit development and ripening. Pomegranate peels are also supposed to contain these classes of active enzymes which need to be quantified based upon ecotypes and commercial varieties to document their antiviral and antibacterial properties during commercial marketing.

Punica granatum L., also known as the pomegranate, is a fruit-bearing, attractive shrub that is commonly grown throughout world. It is most likely of Asian origin, supposedly originated in Iran. Its fruits, which are categorized as berries, have a diameter of 5 to 12 cm and attain a length of up to 14 cm. They have many globular fleshy seeds encased in a sweet, watery pulp that is typically reddish to purple pink colouration. The plant is found all over India. Apart from the fruits' numerous culinary uses, the tea-like aqueous decoction derived from their peels has been employed in conventional medicine to address an extensive array of inflammatory and infectious ailments, including tonsillitis, gingivitis, general viral infections, fungal infections, and respiratory tract ailments [2]. Nanoparticles made from biological molecules are gaining immense popularity and recently histopathological examinations using Berberis lycium Royle-AgNPs engendered significant revival in the morphology of different body parts and suggested it as a potent antidiabetic agent [14]. Antibacterial activity with different sericin-capped silver nanoparticles maintained their bactericidal potentialities and did not affect the efficacy at wide range of temperature and pH [15, 16]. Research also proved that phytoextracts with anthelmintic activity reduced cystoids in mice [17, 18, 19].

During pathological manifestations ROS (e.g., H_2O_2, $O_2^{\bullet}$-, $^1O_2$, $OH^{\bullet}$), accumulation in both living and cancerous cells had been found to reach its peak that would promulgate genomic instability leading to the outrage of malignancy biomarkers [20] and genuine efforts to rescue and to reduce this ROS outburst with the help of phytonutrient-rich plant products have become the safest modes of combating such challenges during clinical trials. Pomegranate had been proved to have anticancer properties through the usage of its juice, peel, and oil. These properties include interference with the growth, invasion, and angiogenesis of malignant cells. These might be connected to pomegranate's anti-inflammatory properties. In *in vitro, in vivo*, and clinical trial treatment of breast, prostate, lung, colon, skin, and hepatic cell malignancies researchers had experimentally shown that pomegranate had anti-proliferative, anti-metastatic, and anti-invasive properties on several cancer cell lines [21]. De Amorim and colleagues (1995) [22] and Valadares *et al.* (2010) [23] examined bone marrow cells for mutagenicity in mice and given an aqueous extract of the fruit's epicarp, they did not detect any significant chromosomal abnormalities. Interestingly, Bouhlali *et al.* (2008) [24] reported that when extracted with ethanol, ripe whole pomegranate fruits, including the peel, would induce varied degrees of genetic changes, including recombination, mutation, aneugenecity and clastogenicity. More recently, Valadares *et al.* (2010) [23] demonstrated using the micronucleus test with an ethanolic extract from pomegranate leaves and fruits had an anti-mutagenic effect against damage generated by cyclophosphamide and did not cause mutagenesis in mice bone marrow cells. Given the conflicting findings of earlier research, addressing the genotoxic potential of preparations generated from this fruit and peels, it became imperative to carry out studies to further illuminate the possible concealments cum hazards associated with improper utilization of pomegranate fruit peel extract both as dietary or investigational tool in *in vitro* models. According to these studies, the fruit's genotoxic potential might vary depending on the phytochemical constitutions, varietal and seasonal variation per say and incidentally the most popularly utilized form in alternative medicine happened to be the aqueous extracts. Thus, in order to gain further insight into the cytotoxic potential of pomegranate peel, this study aims at evaluating the cytotoxic and genotoxic potentials employing principally the aqueous extract from the epicarp of pomegranate fruits. The cytotoxic potentials were analysed through *in vitro* chromosomal aberration

(employing root tip ana-telophase test system) cum nucleolus degradation assays in germinating plant root tip cells (*Lathyrus sativus L.)* and microbial cells *(Escherichia coli, Bacillus sp., Klebsiella sp., Priestia sp.*, and *Rosellomorea maris-flavi*) using agar well diffusion methods.

Among various other spectroscopic methods, we have utilized Fourier Transform Infrared Spectroscopic method, which is widely used in organic synthesis and identification of lead compounds in natural products. The existence of aromatic rings, phenols, alkenes, aliphatic fluoro, alcohols, ethers, carboxylic acids, esters, nitro compounds, and hydrogen bonded alcoholic functional groups were demonstrated by FTIR analysis of *Punica granatum L*. dry fruit peel powder [9]. The chief brainwave behind present study was to evaluate the phytochemical profiling, FT-IR analysis, antibacterial, cytogenotoxic cum apoptosis, antioxidant, anti-haemolytic and anti-tissue damaging activity of aqueous extract from peels of pomegranate fruit that being seasonally available in the markets of Hooghly District of West Bengal, India.

2. Material and methods

2.1. Samples of *Punica granatum* L.

The pomegranate fruits, *Punica granatum* L., Punicaceae family, were collected in the wholesale fruit market of Naihati, North 24 parganas, West Bengal, India in the month of September 2022. The botanical material was identified by Dr. Subrata Mitra Associate Professor and Head of the department of Botany, Hooghly Mohsin College, Chinsurah, Hooghly, West Bengal, India. A voucher specimen was deposited in the departmental herbarium.

2.2. Preparation of the aqueous extract of *Punica granatum* epicarp

Following their harvest, the *P. granatum* fruits were transported to the Hooghly Mohsin College Laboratory PG Department of Botany in Chinsurah, Hooghly, West Bengal, India. There, their seeds were extracted, and the epicarp was spread out on a table and allowed to sit at room temperature in a shaded area. After the epicarp had naturally dried, they were placed in a flask with silica gel to eliminate any last traces of moisture before making the crude aqueous extract. Since the crude extract happened to be the form most often used in traditional medicine, its analysis became crucial. The pulverized epicarp was infused into boiling water at a concentration of 15g/100 ml, boiled at 90°C, and left overnight to produce the aqueous extract using the modified version of a standard method [24]. After cooling to room temperature, the extracts were filtered through cotton fabric to get rid of any leftovers. Every day, just before being used in the tests, fresh *Punica granatum* peel aqueous extract (PGPAE) was prepared, and serial dilutions were made in accordance with protocol. A rotary evaporator was used to concentrate the filtrate at a lower pressure and 40°C until all of the water had evaporated. Before being used, the crude extracts were kept in dark glass bottles at -20°C. To test the extracts' phenol, flavonoid, condensed tannin content, and antioxidant qualities, they were dissolved in distilled water at a known dilution.

2.3. Phytochemical screening of *Punica granatum* peel aqueous extract (PGPAE) (Qualitative)

Phytochemical Screening Methods: Using routine chemical tests, the main phytochemical ingredients in aqueous extracts were verified.

The following tests were performed-

a. Test for tannins and phenolic compounds (ferric chloride test): About 0.5 cc of test solution was placed in a test tube and a few drops of ferric chloride were added.
b. Test for flavonoids: Magnesium hydro chloride reduction test, or Shinoda test: A little amount of magnesium ribbon reagent and dropwise additions of strong hydrochloric acid were made to test the solution (0.5–1 ml).
c. Test for saponins (foaming test): Added roughly 0.5 ml of extract to 5 ml of distilled water, then given it a good shake. Five minutes were given to the mixture to stand.
d. Test for proteins (Ninhydrin test):A test tube containing approximately 0.5–1 millilitres of material was filled and heated with a 0.2 molar solution of ninhydrin. The test sample contained aromatic proteins if a violet tint would develop.
e. Test for steroids and terpenoids (Salkowski test):In a test tube, 0.5–1 millilitres of the test solution were

chloroformed. After adding a few drops of strong sulfuric acid and giving it a good shake, some time was allowed to pass.

f. Test for alkaloids (Mayer s test): In a test tube, 0.5–1 ml of the sample was obtained. After adding a few drops of Mayer's reagent and giving it a good shake, it was allowed stand for a while.

g. Test for carbohydrate (Benedict's test):Poured two millilitres of Benedict reagent in a test tube. Slowly, five drops of the 0.5 percent test solution were added.

2.4. Quantitative estimation of some major phytochemical compounds and enzymatic constituents in the *Punica granatum* peel aqueous extract (PGPAE)

2.4.1. Quantification of total phenolics in *Punica granatum* peel aqueous extract (PGPAE)

The total phenolic content of plant extracts was determined by applying a standard approach [25]. In summary, 1 millilitre of the extract was combined with 5 millilitres of a 1/ 10 dilution of the Folin-Ciocalteu reagent in distilled water. Afterwards, 4 millilitres of a 7.5% w/v sodium carbonate solution were added. After the combination was allowed to settle at room temperature for 60 minutes, the absorbance at 765 nm was measured. A calibration curve was created using gallic acid. The amount of total phenolic compounds per gram of plant dry weight (DW) was represented in milligrams of gallic acid equivalent (GAE).

2.4.2. Quantification of total flavonoid content in *Punica granatum* peel aqueous extract (PGPAE)

With only minor adjustments, the Kim *et al*. (2021) [26] method was employed to ascertain the total flavonoid content of the plant. Four millilitres of distilled water were combined with one millilitre of plant extract. Next, 0.3 millilitres of a sodium nitrite solution and 0.3 millilitres of a 10% aluminium chloride solution were added. Five minutes were spent letting the mixture lie at room temperature before adding 2 mL of sodium hydroxide (1 M) and the remaining volume being made up of 10 mL of distilled water. After gently vortexing the liquid, the absorbance at 510 nm was determined. The results were expressed after the readings were adjusted to a predetermined routine standard curve as milligrams of gallic acid equivalent (GAE) per gram of dry weight (DW).

2.4.3. Quantification of total condensed tannins in *Punica granatum* peel aqueous extract (PGPAE)

The method of Heimler *et al.* (2006) [27] was adjusted to determine the total condensed tannin concentration. To summarize, 400 µL of the plant extract, 1.5 mL of strong hydrochloric acid, and a methanolic solution of vanillin (4%) in ethanol were combined. After 15 minutes of room temperature incubation, the mixture's absorbance at 500 nm was measured. Drawing from a catechin calibration curve, the results were expressed as mg CE (Catechin Equivalent)/g DW (plant dry weight).

2.5. Extraction and estimation of peroxidase and polyphenol oxidase from pomegranate fruit peel

Pomegranate (*Punica granatum*) fruit peel fragments were freshly gathered in minute pieces for the purpose of extracting and estimating the enzymes peroxidase and polyphenol oxidase. With very minor adjustments, these enzymes were isolated using the $(NH_4)_2SO_4$ purification process and calculated using the techniques described by Ghosh and Basu (2006) [28]. Based on Lowry *et al.* (1951) [29], the protein content of the enzyme extract was calculated. Peroxidase and Polyphenol oxidase were estimated by allowing the reaction to run for five, ten, and fifteen minutes.

2.6. FT-IR analysis of dry pomegranate fruit peel powder

FT–IR (determining the Fourier transformation Infrared) spectroscopy is a valuable tool used to obtain specific information about the chemical bonds and molecular structure of bulk polysaccharides and other major phyto-chemicals present [30] in the dry peel powder. The main absorption bands are given in cm^{-1}.

Spectroscopy Instrument: Recording of IR spectra was carried out and recorded on a Fourier transform IR spectrophotometer (Bruker Alpha II FTIR Spectrometer).

Procedure: The sample used for the spectral analysis was solid, so it was crushed using an oily mulling agent to create a thin layer. Finally, a tiny layer of this mull was put to a salt plate for the measurement.

2.7. Antimicrobial assays: Effect of *Punica granatum* peel aqueous extract (PGPAE) on microbial cultures

For the antimicrobial study strains of *Escherichia coli, Bacillus sp., Klebsiella sp., Priestia sp., and Rosellomorea maris-flavi* were selected to see the antimicrobial effect of *Punica granatum* peel aqueous extract (PGPAE).

2.7.1. Preparation of different strengths of *Punica granatum* peel aqueous extract (PGPAE) and standard antibiotics

Antimicrobial activity of pomegranate fruit peel extract against selected microorganisms was examined. Test extract (10 g/100 ml) was diluted to obtain different concentrations. First the serial ½ dilution was done to get extract with concentration 5 g/100 ml. About 1 ml of 5 g/100 ml solution was taken and 1 ml of distilled water was added to it. To obtain concentration 2.5g/100 ml 1 ml of 10 g/100 ml extract was added to 3 ml of distilled water. Each prepared solution having different strength was pour into each respective well of each respective plate. By serial dilution method various concentrations of chosen drugs, Penicillin and Amoxycillin were prepared. For preparation of different doses of penicillin drug, in 1st tube mother stock solution of 400 mg/ml was taken. About 1 ml of mother stock solution was taken to 2nd test tube to which 1 ml of sterilized distilled water was added to make a drug dose of 200 mg/ml. Next for 100 mg/ml dose, 1 ml of solution was taken from 2nd test tube into 3rd test tube and 1 ml of sterilized distilled water was added to it. For 50 mg/ml dose preparation, 1 ml solution was taken from the 3rd tube and added to sterilized distilled water in a 4th tube. *E. coli* and *Priestia* sp., bacteria were then treated with these prepared doses. Different doses of Amoxycillin were prepared from mother stock solution of concentration 500 mg/ml taken in 1st test tube. To 2nd test tube 0.1 ml of mother stock solution was taken and 0.9 ml sterilized distilled water was added to it making a dose of 50 mg/ml. In 3rd tube 1 ml of mother stock solution was taken and 1 ml of sterilized distilled water was added to get 250 mg/ml concentration. About 1 ml solution from 3rd test tube was taken in 4th tube to which 1 ml sterilized distilled water was added, thus 125 mg/ml dose was prepared. Antibacterial activity of Amoxycillin drug was observed using *E. coli* and *Priestia* sp. bacteria.

2.7.2. Effect of *Punica granatum* peel aqueous extract (PGPAE) on different bacteria

Both Gram-positive (*Bacillus* sp., *Priestia* sp., and *Rosellomorea maris-flavi*) and Gram-negative (*Escherichia coli*) bacteria were selected for the study. Authentic bacterial strains were collected from the microbiology laboratory of the university of Burdwan and Barasat Government College, West Bengal State University. The bacterial strains were used for experiments after regular routine subculturing (250 days). To test the antimicrobial effect of fruit peel of pomegranate agar cup assay method was performed following Miller and Rose (2015) [31] with little modification. Nutrient agar plates were inoculated with the bacterial strains and kept it for 10-15 minutes. Then the plates were divided into four equal parts. Then with the help of a sterile cork-borer,4 cups or cavities were made in each portion. Each cavity or cup was then filled with of *Punica granatum* peel aqueous extract (PGPAE) of different concentrations mentioned above against a control set with sterile distilled water. The antimicrobial activity of *Punica granatum* peel aqueous extract (PGPAE)was compared with the standard antibiotics (Penicillin and Amoxycillin) following the same assay method. Then the plates were incubated with 35±2°C for 24 hours. The zones of inhibitions against of the each concentration of *Punica granatum* peel aqueous extract (PGPAE) was measured after incubation and the data are plotted in a graph paper.

2.8. Cytogenotoxic activity of *Punica granatum* peel aqueous extract (PGPAE)

Determination of cytogenotoxicity of *Punica granatum* peel aqueous extract (PGPAE) (% of Mitotic inhibition in germinating root tips of *Lathyrus sativus* L., variety Mahatora aby orcein staining): State Seed Testing Laboratory, District Burdwan provided the dried seeds of *Lathyrus sativus* L., variety Mahatora. Following many thorough washes in distilled water, around ten seeds were incubated for twenty-four hours using the aforementioned dilutions (a process known as "seed priming of *Punica granatum* peel aqueous extract, or PGPAE). After multiple

trials, it was shown that after five days of incubation at room temperature, seeds that had been soaked with 10 and 7.5 g/100 ml began to germinate. With all healthy morphological characteristics, seed priming at a rate of 5 g/100 ml to 0.625 g/100ml could promote healthy root emergence and prevent blackening of the root tips. For the *Lathyrus sativus* L., chromosomal aberration assay and *Lathyrus sativus* L. nucleolar index (NI) study experiment, the treatment regimens that resulted in a scorable Mitotic Index (MI), sufficient Chromosomal Aberrations (changes in MI%) in meristematic cells, as well as observable alterations in nucleolar Index percentage (NI%) and volume, were chosen.

In order to investigate the cytogenetic alterations brought about by *Punica granatum* peel aqueous extract (PGPAE) at serially diluted doses in the *Lathyrus sativus* L. plant, mitotic cells were obtained from the root tips of sprouted seeds [32]. The mitotic index (MI) and chromosomal aberrations in metaphase and anaphase plates were inspected under high power (40x) an oil immersion objective (100x). A minimum of 100 cells were scored from each slide, and the mitotic index (MI) was calculated [32]. On each slide, chromosomal anomalies were analyzed in a minimum of 100 cells and reported as a percentage. These included chromosomal fragments (F), premature separation (SP), stickiness (STC), bridge formation (Br), c-mitosis (C-m), micronuclei, somatic triploid and tetraploid, unequal separation, precociousness, nuclear budding and fragmentations, strap cells and giant ghost cells, karyorrhexis and karyolysis, margination, etc. Using a 100 × eyepiece on an Olympus CH20i compound microscope equipped with a CMOS camera (IS 500, 5.0 MP) and its computer connection with the help of VIEW 7 image processing software, all phases were inspected at 40 x and under oil immersion objective [33, 34].

Images were acquired after cytotoxic and genotoxic end-point parameters (i.e., Mitotic Index %, Genotoxicity Index (GnI); GnI%, Mitotic Inhibition Percentage and frequency of micronuclei; (FMN), where MN served as a biomarker for cytogenotoxicity) were calculated using formulas [32].

2.8.1.Detection of morphological characters for cell death

Computation the Percentage of Dying Cells: We chose nucleus migration from centre to margin of cell wall, condensation, vacuolation of cytoplasm, nuclear fragmentations as distinctive markers of dying cells. The displacement of the nucleus in a cell wall margin is known as nucleus margination [35].

$$PDC = \frac{\text{No. of cells dying or dead cells}}{\text{Total no. of cells counted}} \cdot 100$$

where PDC - Percentage of Dying Cells

2.8.2.Measurement of cell dimensions of Giant cells with vacuolated cytoplasm

Roots were placed in an incubation solution (0.1 M sodium phosphate buffer pH 7.2, 0.3 M sucrose, and 6 µM neutral red) for 30 minutes in order to measure the amount of protoplasmic shrinkage in germinating root cells [32]. After hydrolyzing the cell roots in 1N HCl, safranine solution was used as a counter stain. The root tips were then squished in 45% acetic vinegar and Photographs were taken from all the morphogenic zones of the root tip (in 100X magnification) and cell dimensions like shrinkage areas, length, width and cytoplasmic areas were measured [35].

2.8.3.Computation of nucleocytoplasmic ratio (NPR): Cytoplasm surface were measured in photographs and NPRs were computed in each cell with ocular micrometre [35].

$$\text{NPR}=\frac{\text{average reduction in the nucleus area of the dying cells}}{\text{average reduction of cytoplasm of the dying cells + giant cells}} \cdot 100$$

[32].

2.9. Effect of PGPAE to evaluate nucleolar frequency and nucleolar index in root tip cells using Haematoxylin staining

After 24 hours of varying quantities of test sample (*Punica granatum* peel aqueous extract; PGPAE) pre-exposure (seed priming), seeds of the *Lathyrus sativus* L variety Bidhan Khesari were allowed to germinate. The root tips were then cut and fixed in FAA after 72 hours of germination. The following day, the root tips were hydrolyzed for 45 min. in a water bath containing 45% acetic acid, without allowing the temperature to increase over 85°C. After acid hydrolysis the root tips were cooled, rinsed in distilled water and treated with iron alum (ferric ammonium sulfate) 10% solution for 10 minutes followed by staining in 2% aqueous haematoxylin solution for 45 minutes. In each set of the experiment, one

thousand bi-nucleolated cells with intact cytoplasm were scored each coded slide in order to assess the induction of micro-nucleoli. The following criteria were used to identify bi-nucleolated cells with micronucleoli: Micronuclei containing micronucleoli should be smaller than one-third of the main nuclei; Micronuclei must not touch the main nuclei; Micronuclei must not be refractive and should be the same colour and intensity as the main nuclei; Both nuclei and micronuclei containing the nucleolus should be rounded. Every experimental point's nucleolar division index (NDI) was determined. For each culture, five hundred live cells were scored in order to compute the NDI using the formula and find the average of the cells with 1, 2, 3, 4 and 5 nuclei bearing conspicuous nucleoli [32]:

$$NDI = \frac{1(\mathrm{M1}) + 2(\mathrm{M2}) + 3(\mathrm{M3}) + 4(\mathrm{M4}) + 5(\mathrm{M5})}{N}$$

where, NDI - nucleolar division index, M1-M5 represent the number of cells with 1 to 5 micro-nuclei having prominent micro-nuclei and N is the total number of viable cells scored. The respective NDI were then converted to NDI %. The results of the three independent tests were totalled and statistically analyzed.

2.10. Antioxidant profiling of PGPAE

2.10.1. Scavenging capability for 1,1-diphenyl-2-picrylhydrazyl(DPPH)

Radicals by PGPAE: The capacity to scavenge free radicals using the approach outlined in Shimada *et al*., 1992 [36] had been utilized here. The free radical scavenging activity of PF-1, PF-2, and PF-3 (of PGPAE) were assessed in the concentration range of 25-200 µg/mL using a stable DPPH* and butylated hydroxy anisole (BHA) as standards. The volume of each sample solution concentration was equalized after addition of 1 mL of freshly prepared methanolic DPPH solution (0.5 mM) and well mixed. After that, it was left to stand in a dark area at 37^{o}C for an hour. The absorbance of DPPH• and the sample solution were measured at 518 nm relative to a blank. To determine PGPAE's ability to scavenge the DPPH* during each triplicate, the following formula was employed:

$$DPPH\ scaveging\% = \frac{A_{blank} - A_{sample}}{A_{blank}} \cdot 100$$

where, A_{blank} is the absorbance value of the control reaction and A_{sample} is the absorbance value of the extract.

2.10.2. Ferric ion reducing/antioxidant power (FRAP) assay by PGPAE

Pulido *et al*. (2000) [37] revised the FRAP experiment technique based on work by Benzie and Strain (1996) [38]. To put it briefly, the oxidant in 40 mM HCl was prepared by mixing 25 mL of 0.3 M acetate buffer (pH 3.6), 2.5 mL of 20 mM $FeCl_3$, $6H_2O$, and 2.5 mL of a 10 mM TPTZ [2,4,6-tri(2-pyridyl-5-triazine) solution. The final solution had TPTZ of 0.83 mM and Fe(III) of 1.67 mM. After heating 900 µL of freshly manufactured FRAP reagent to 37°C and taking a reagent blank reading at 593 nm, 30 µL of the test sample and 90 µL of distilled water were added. Absorbance measurements were acquired after 0.5 seconds and then every 15 seconds for 30 minutes. Using a Shimadzu UV-1601 (PC) Spectrophotometer, absorbance readings were collected after 0.5 s and then every 15 s till 30 min. The absorbance change ($\Delta A = A_{30min} - A_{0min}$) was computed related to the ΔA of Fe(II) standard. Calibration was performed using aqueous solutions with known Fe(II) concentrations (100–2000) µM/L $FeSO_4$, $7H_2O$. BHT, or butylated hydroxytoluene, served as the reference material.

2.11.Anti-haemolytic (against H_2O_2 induced ROS) and anti-tissue damaging activity of PGPAE

- With a few small adjustments, the previously published procedure [39] was used to screen the anti-haemolytic activity of PGPAE. Fresh chicken blood was procured from nearby slaughterhouses and placed in heparinized centrifuge tubes for testing purposes. The blood was separated via centrifugation and then promptly cleaned with phosphate buffer (pH 7.4) before being diluted with phosphate buffered saline to create a 2% solution. About 2 ml of the erythrocyte suspension were mixed with different concentrations of PGPAE (100, 200, 250, 500, 1000, 2000 and 4000 µg/ml diluted in Phosphate Buffered Saline), and the volume was increased to 4 ml using phosphate buffer (pH 7.4). After allowing the mixture to sit at room temperature for five minutes, 0.5 ml of a saline buffered-H_2O_2 solution was

added to cause the membrane lipids to oxidatively degrade the RBC membrane. The reaction mixture's H_2O_2 content was so adjusted to produce about 50% haemolysis of the RBCs after 60 minutes of incubation at 37°C. The reaction mixture was centrifuged for 10 minutes at 2000 rpm after being incubated for 60 minutes at 37°C. The amount of haemolysis was determined by measuring the absorbance at 540 nm, which indicated the liberation of haemoglobin. For the purpose of converting O.D. vs. % haemoglobin released by PGPAE against positive (distilled water; regarded as 100% lysis) and negative control (phosphate buffer saline). The particular PGPAE concentrations were divided into three main groups according to the inhibition: The samples were divided into three categories: (a) neutral, where the amount of haemolysis was almost the same as the positive control; (b) weak, where the percentage of haemolysis inhibition was less than 20% of the positive control; and (c) potent, where the percentage of haemolysis inhibition was less than 80% of the positive control. To assess the anti-haemolytic activity of PGPAE (50, 100, 250, 500, 1000, 2000, and 4000 µg/ml in Phosphate Buffered Saline) on human erythrocytes *in vitro,* the identical protocol mentioned above was used. About 5 ml of blood were drawn from a healthy, normal person and centrifuged for three minutes at 1500 rpm. Three rounds of washings were performed on a blood pellet in sterile phosphate buffer saline solution (pH-7.4). The Helsinki Declaration was adhered to by the study protocol.

- With minor modifications fresh chicken hearts, livers, and lungs were cut into uniformly-sized small pieces, cleaned with a 0.9% NaCl solution, and then patted to dry with tissue paper to test for *in vitro* anti-tissue damaging activity of PGPAE. The 350±10 mg tissue pieces were pre-incubated for 45 minutes at 37°C in 1 mL of 200 mM potassium-phosphate buffer (pH 7.4). After two washes with the same solution, the tissue strips were incubated for five hours at 37°C in a volume of three millilitres (in 200 mM K-phosphate buffer, pH 7.4) with a fixed concentration of PGPAE (100, 200, 250, 500, 1000, 2000, and 4000 µg/ml in PBS). After incubation for four hours, the reaction mixtures were centrifuged for five minutes at 2000 rpm. The absorbance of the supernatant at 540 nm was measured using a Hitachi U-2000 spectrophotometer. According to Adhikari and Karmakar (2018) [40], tissue samples treated in 0.2% Triton X100 solution-which were considered to generate 100% lysis in standard sets were extrapolated to calculate the percentage of haemoglobin liberated.

2.12. Statistical Analysis

Each and every value is shown as Mean ± SD (standard deviation, n=6). Analysis of variance (ANOVA) was used in the statistical analyses, which were then followed by the "t" test, Brown-Forsythe, and Welch's Post-hoc tests, whichever is demanded, to determine the significance level of each group's mean compared to the control. P values deemed significant were those that were less than 0.05. In the statistical study, the GRAPH PAD PRIZM-version 8 computer program's analysis of variance (ANOVA) was used to examine differences between the groups.

3. Results

The phytochemical screening (qualitative) revealed the strong presence of carbohydrates, proteins, cardiac glycosides, tannins, phenolic compounds, flavonoids, saponins, and terpenoids.

3.1. The phytochemical screening (qualitative) of carbohydrates, proteins, cardiac glycosides, tannins, phenolic compounds, flavonoids, saponins, and terpenoids

The phytochemical screening (qualitative) revealed the strong presence of carbohydrates, proteins, cardiac glycosides, tannins, phenolic compounds, flavonoids, saponins, and terpenoids (Table 1).

3.2. Quantitative estimation of total phenolics, flavonoids and condensed tannins from PGPAE

The quantitative estimation processes revealed the strong presence of phenolics, flavonoids and condensed tannins in PGPAE out of which polyphenols and flavonoids are maximum followed by Tannis (Table 2).

Table 1. Qualitative phytochemical screening of PGPAE with major phytochemicals present

Phytochemical constituents tested	Presence/ absence in PGPAE	Confirmatory test
Carbohydrates	+	A red precipitate resulted from reducing sugars.
Proteins	+	The appearance of violet color indicated the presence of protein.
Cardiac glycosides	+	Cardiac glycosides were present when the color turns green.
Tannins	+	The sample's appearance of blue-green color indicated the presence of tannins.
Phenolic compounds	+	The sample's presence of phenolic compounds was confirmed by the appearance of blue-green color.
Flavonoids	+	After a few minutes, crimson red coloration emerged, indicating that flavonoids were present in the sample.
Saponins	+	Saponins were found in the sample, indicating persistence of frothing.
Steroids	–	Absence of redness at the bottom layer, which would suggest the presence of steroids
Terpenoids	+	The presence of terpenoids was indicated by the appearance of the yellow layer.

Table 2: Quantitative estimated values of phenolics, flavonoids and total condensed tannis were expressed as in mg GAE/g dry weight of pomegranate peel powder

Total phenolic content of PGPAE (mg GAE/g dry weight)	Total flavonoid content of PGPAE (mg GAE/g dry weight)	Total Condensed Tanin content CE (Catechin Equivalent)/g dry weight
10.23±1.2	7.15±0.84	3.22±0.54

3.3. Extraction and quantitative estimation of total peroxidase and polyphenol oxidase in fresh pomegranate fruit peel

The peel extract of pomegranate fruit contained a large amount of peroxidase (max. 161.26 µg purpurogallin formed / mg protein / min after 15 minutes of reaction time) and polyphenol oxidase (max. 138.53 µg purpurogallin formed / mg protein / min after 15 minutes of reaction time). The amount of enzymes was different for both peroxidase and polyphenol oxidase allowing the reaction time from 5 min to 15 min. Thus the fresh peel of pomegranate was containing a rich source of these two vital enzymes (Table 3).

Table 3: Extraction and quantitative estimation of total peroxidase and polyphenol oxidase in fresh pomegranate fruit peel

Material	Reaction Time	Mean Peroxidase activity (µg purpurogallin formed/mg protein/min)	Mean Polyphenol oxidase (µg purpurogallin activity formed/mg protein/min)
Fruit peel extract of Pomegranate	5 Min	154.44	134.30
	10 Min	159.98	137.26
	15 Min	161.26	138.53
Critical difference at $P = 0.05$		1.32	1.06

3.4. FT-IR analysis of dry pomegranat fruit fruit peel powder

The FTIR spectra obtained from the peel powder extract of *Punica granatum* L. dry fruit has been shown in Fig. 1. The characteristic peaks of phytochemicals present in *Punica granatum* L. dry fruit of C-H stretching of alkene, OH- group of alcohol, amine, C-H stretching of alkane, the bands for deprotonated -COOH group and N–H bond of primary amide and O–H stretching of polyphenols as reported in literature [41, 42, 43] are shown at 584, 778, 885, 1314, 1440, 1608 and 3322 cm^{-1}. This spectrum depicts that *P. granatum* L. dry fruit peel extract contains different phytochemicals that have O–H groups as like flavonoids and phenols. Moreover, from spectra, the presence of

functional groups such as alcohol, carboxylic acid, ester and ether was confirmed in the *P. granatum* L. dry fruit peel extract. The earlier reports had shown that the existence of several chemical component families, including phenols (cyclic compounds) (OH Ar-OH, C_6H_6, C-O-C ether oxide, C-N, C-O phenol and C=O) using FTIR spectroscopy to analyze the phytochemical composition of pomegranate peel powder. Indeed, the antioxidant qualities of this fruit are mostly due to the presence of certain phenolic acids in plant extracts, such as punicalagin, ferulic acid, and p-coumaric acid. According to Medic-Saric *et al*. (2004) [44], punicalagin is a hydrolysable tannin and o-coumaric acid that is found in frequencies between 3200 and 3600 cm^{-1}. Laoufi (2017) [45] found similar results, noting the existence of C=C (1636.52 cm^{-1}), O-H groups (3306.30 cm^{-1}), and an elongation of the C=O bond. Additionally, functional clusters were identified which included the following: 3400 cm^{-1} (OH) function, 2940 cm^{-1} (OCH) liaison, 2840, 2805 cm^{-1} (OCH), 1253 cm^{-1} (furan), 1235 and 1035 cm^{-1} (lignan), and between 1630-1550 cm^{-1} (aromatic). The present study's findings happened to be similar to those of Sehari *et al.* (2022) [30], who shown that certain phenolic acids, including o-coumaric acid and ferulic acid, were present in plant extracts at frequencies ranging from 3200 to 3600 cm^{-1} (Fig. 1).

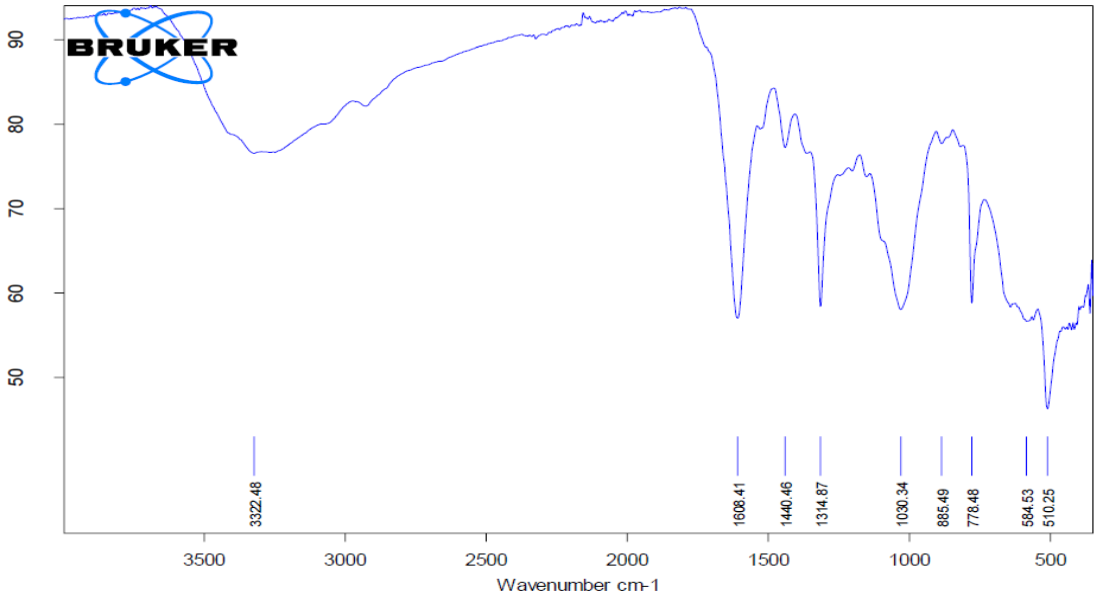


Figure 1. Fourier Transform Infra-Red (FT-IR) spectroscopic analysis of the major peaks from the dried fruit peel powder of *Punica granatum* L.

3.5. The effect of PGPAE on different microbial strains

The effect of PGPAE on different microbial strains (the antimicrobial activity) is presented in the table 4.

The antimicrobial activity of PGPAE tested on the bacterial strain *Bacillus* sp. exhibited maximum inhibition (1.2 cm) to occur when the agar cup was filled with maximum dose (10g/100ml). The bacterial strain (*Bacillus* sp.) tested for different concentration of the PGPAE augmented gradual inhibition in the form of inhibitory zone in agar plate which could be due to the sensitivity of the different phytochemicals, present within the extract. It was found that after the concentration (5g/100ml) there was no further increase of the diameter of the inhibitory zone. So it can be concluded that the minimum inhibitory concentration of PGPAE for maximum inhibition of bacteria *Bacillus* sp. was 5g/100ml. In view to compare the PGPAE with standard antibiotics (penicillin, amoxicillin) the microorganism *Bacillus* sp. demonstrated maximum sensitivity against the antibiotic Penicillin and Amoxicillin at 200mg/ml and 400mg/ml respectively. Though the lower concentration of the antibiotic Penicillin (50mg/ml) and Amoxicillin (125 mg/ml) produced inhibition in the form of inhibitory zone on agar cup which were 1.5 cm and 1.2 cm respectively but the inhibitory zone formed due to the use of PGPAE at higher

concentration (10g/ml)

Table 4 Effect of PGPAE on different microbial strains (anti-microbial activity)

Bacterial strain	PGPAE strength (zone of inhibition in cm) (10g/100ml)	PGPAE strength (zone of inhibition in cm) (5g/100ml)
Priestia sp.	1.45 cm	1.35 cm
Rosellomorea maris-flavi	0.6 cm	0.6 cm
Escherichia coli	1.45 cm	1.15 cm
Bacillus sp.	1.3 cm	1.25 cm
Klebsiella sp.	1.15 cm	0.6 cm

Strain	Control	¼ diluted (2.5 g/ 100ml)	½ diluted (5 g/ 100ml)	Concentrated (10 g/100ml)
Bacillus sp.	0	1 cm	1.2 cm	1.2 cm
Priestia sp.	0	0.6 cm	1.3 cm	1.3 cm

For Penicillin treatment

Strain	Control	50 mg/ml	100 mg/ml	200 mg/ml	400 mg/ml
Bacillus sp.	0	1.5cm	1.6cm	1.9cm	1.9cm
Priestia sp.	0	0.6cm	1.5cm	2cm	2cm

For Amoxicillin treatment

Strain	Control	50 g /ml	125 g /ml	250 g /ml	400 g /ml	500 g/ml
Bacillus sp.	0	0.6 cm	1.2 cm	1.7 cm	1.9 cm	1.9 cm
Priestia sp.	0	0	0	0	0	0

was 1.2 cm. It was obvious that the antibiotics used had the capacity to inhibit the bacteria *Bacillus* sp. was high in comparison to the PGPAE. Still the phytochemicals within PGPAE exhibited the capacity to inhibit the growth of *Bacillus* sp. thus promulgating its antimicrobial properties. The antimicrobial activity of PGPAE on bacterial strain *Priestia* sp., was also established. The result obtained in agar cup assay demonstrated the bacterial strain *Priestia* sp., had maximum inhibitory zone of 1.3 cm when treated with concentrated extract (10g/100ml). Against different concentrations of PGPAE, the strain *Priestia* sp., exhibited gradual inhibition of bacterial growth measured as inhibitory zones on agar plate. It was observed that at concentration 5g/100ml the inhibition was of 1.3 cm and no further increase in inhibitory zone was found thereafter. So for *Priestia* sp., the maximum inhibition was 1.3 cm with minimum inhibitory concentration 5g/ml. Comparing PGPAE with standard antibiotics (Penicillin and Amoxicillin) the bacteria *Priestia* sp., augmented maximum sensitivity for Penicillin at 100mg/ml dose with a zone of 1.5cm (Fig. 2). Amoxicillin treatment did not show any activity against *Priestia* sp. This is can be due to the evolutionary changes in the bacteria species *Priestia* sp., probably making it resistant to the antibiotic Amoxicillin.

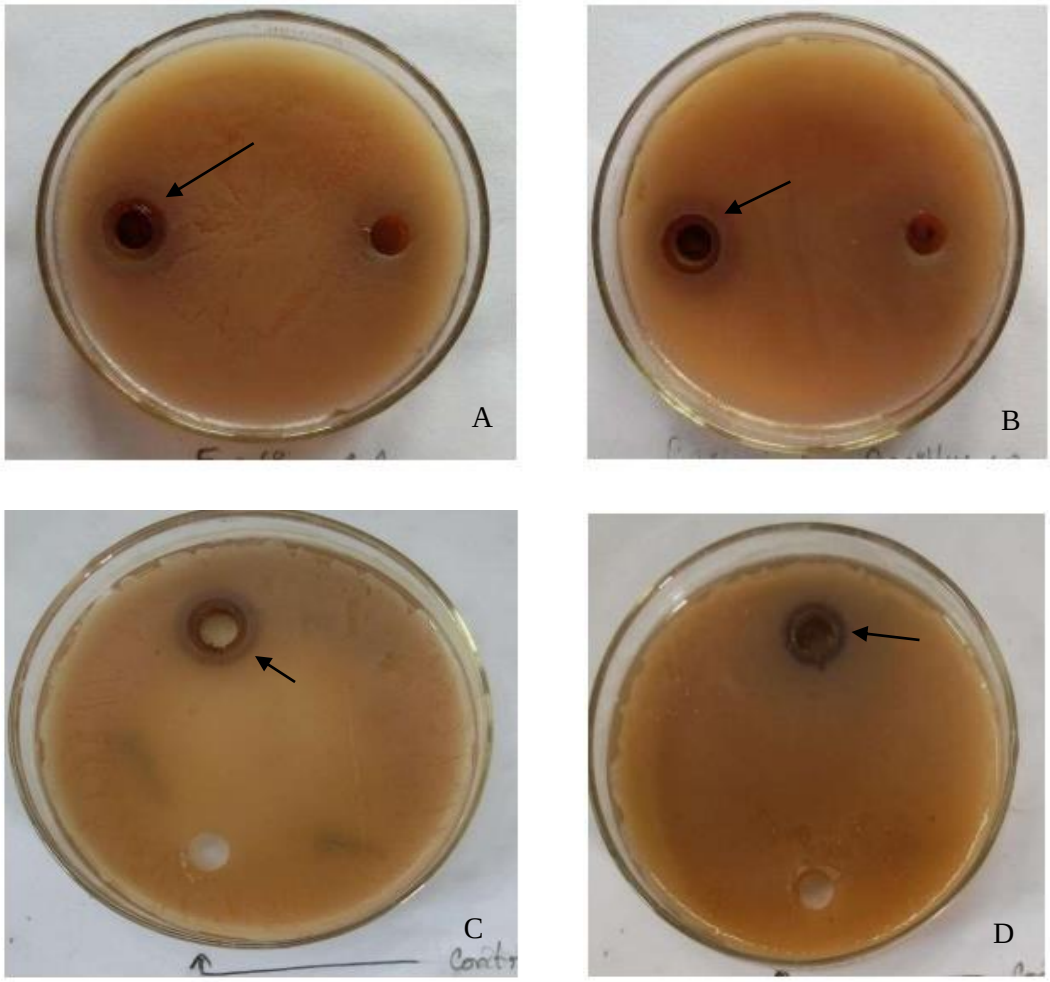


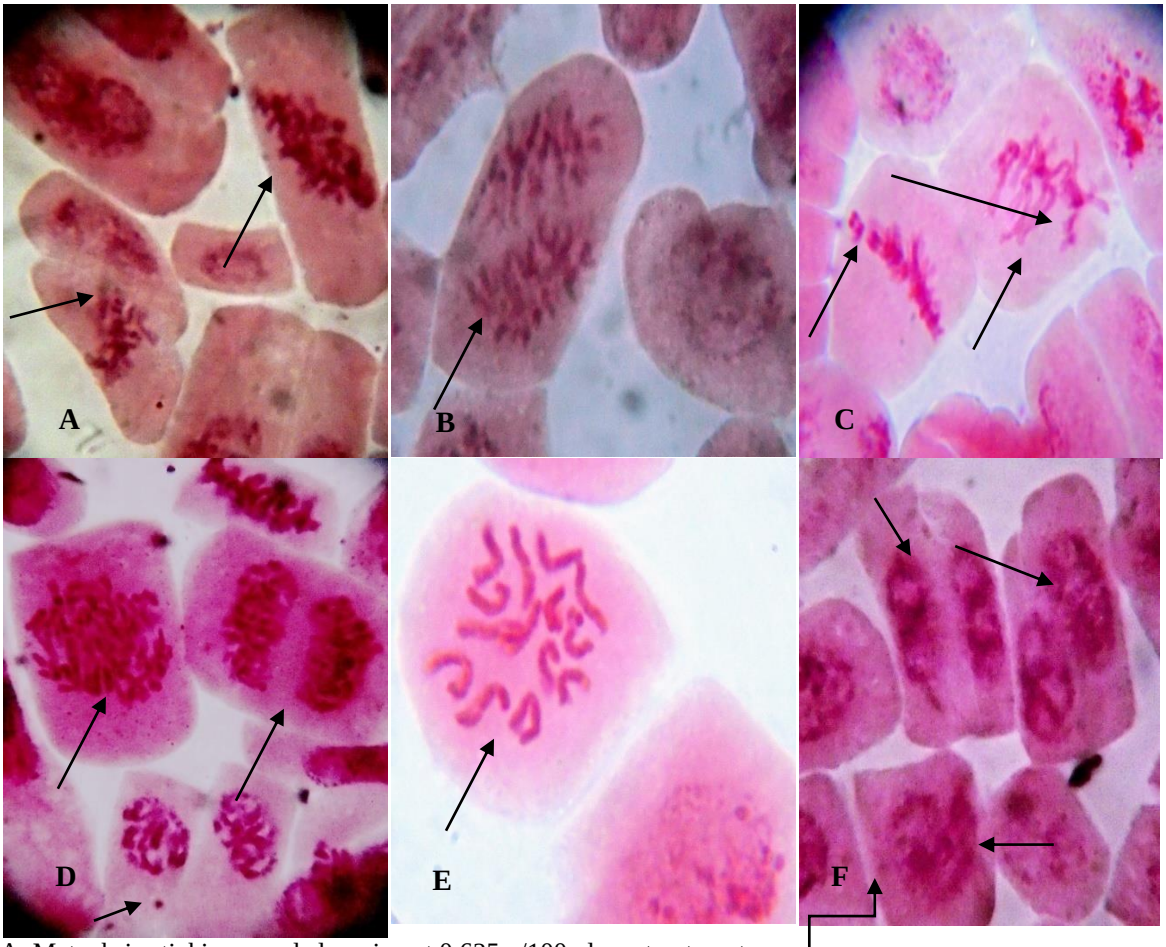
Figure 2 (Plates A-D): Antimicrobial activity of PGPAE against four different genera of bacteria. A: diameter of zone of inhibition of PGPAE (10g/100ml) against *Escherichia coli, B:* diameter of zone of inhibition of PGPAE (10g/100ml) against *Bacillus* sp., C: diameter of zone of inhibition of PGPAE (10g/100ml) against *Priestia sp., D:* diameter of zone of inhibition of PGPAE (10g/100ml) against *Klebsiella* sp.

The antimicrobial activity of PGPAE tested on the bacterial strain *Bacillus* sp. exhibited maximum inhibition (1.2 cm) to occur when the agar cup was filled with maximum dose (10g/100ml). The bacterial strain (*Bacillus* sp.) tested for different concentration of the PGPAE augmented gradual inhibition in the form of inhibitory zone in agar plate which could be due to the sensitivity of the different phytochemicals, present within the extract. It was found that after the concentration (5g/100ml) there was no further increase of the diameter of the inhibitory zone. So it can be concluded that the minimum inhibitory concentration of PGPAE for maximum inhibition of bacteria *Bacillus* sp. was 5g/100ml. In view to compare the PGPAE with standard antibiotics (penicillin, amoxicillin) the microorganism *Bacillus* sp. demonstrated maximum sensitivity against the antibiotic Penicillin and Amoxicillin at 200mg/ml and 400mg/ml respectively. Though the lower concentration of the antibiotic Penicillin (50mg/ml) and Amoxicillin (125 mg/ml) produced inhibition in the form of inhibitory zone on agar cup which were 1.5 cm and 1.2 cm respectively but the inhibitory zone formed due to the use of PGPAE at higher concentration (10g/ml) was 1.2 cm. It was obvious that the antibiotics used had the capacity to inhibit the bacteria *Bacillus* sp. was high in comparison to the PGPAE. Still the phytochemicals within PGPAE exhibited the capacity to inhibit the growth of *Bacillus* sp. thus promulgating its antimicrobial properties. The antimicrobial activity of PGPAE on bacterial strain *Priestia* sp., was also established. The result obtained in agar cup assay demonstrated the bacterial strain *Priestia* sp., had maximum inhibitory

zone of 1.3 cm when treated with concentrated extract (10g/100ml). Against different concentrations of PGPAE, the strain *Priestia* sp., exhibited gradual inhibition of bacterial growth measured as inhibitory zones on agar plate. It was observed that at concentration 5g/100ml the inhibition was of 1.3 cm and no further increase in inhibitory zone was found thereafter. So for *Priestia* sp., the maximum inhibition was 1.3 cm with minimum inhibitory concentration 5g/ml. Comparing PGPAE with standard antibiotics (Penicillin and Amoxicillin) the bacteria *Priestia* sp., augmented maximum sensitivity for Penicillin at 100mg/ml dose with a zone of 1.5cm (Fig. 2). Amoxicillin treatment did not show any activity against Priestia sp. This is can be due to the evolutionary changes in the bacteria species *Priestia* sp., probably making it resistant to the antibiotic Amoxicillin.

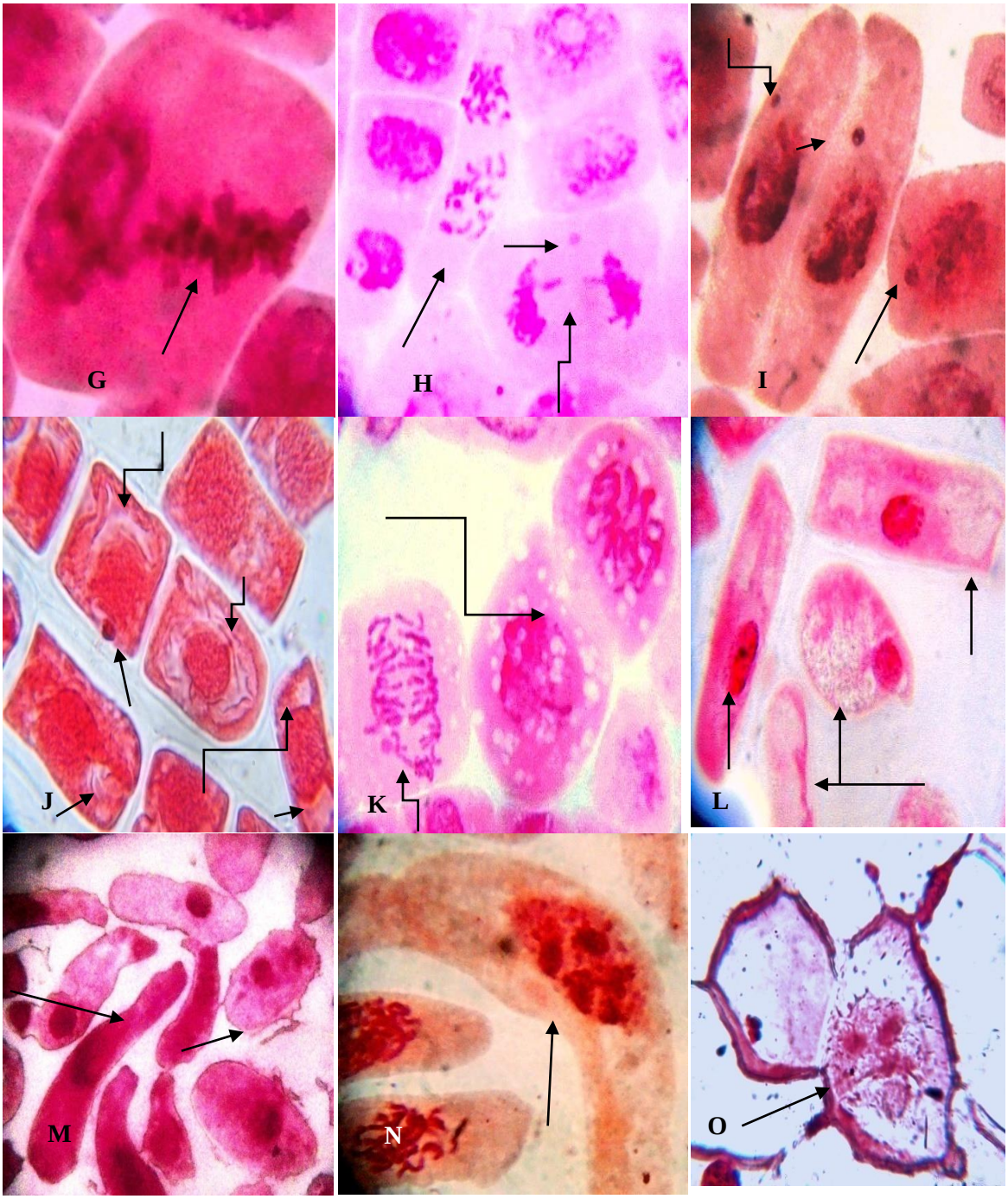
3.6. Cytogenotoxicity analysis

The determination of cytogenotoxicity of PGPAE (% of Mitotic inhibition in germinating root tips of *Lathyrus sativus* L., variety Mahatora by orcein staining) is presented in the figure 3.



A: Metaphsic stickiness and clumping at 0.625 g/100ml pre-treatment,
B Tropokinesis at1.25 g/100ml;
C: Pole to pole metaphasic stickiness and dislocated chromatid fragments at metaphase at 2.5 g/100ml,
D: polyploidy formation and coagulated anaphasic chromosomes 2.5 g/100ml,
E: C-mitosis at 2.5 g/100 ml;
F: giant cells with multivacuolated somatic diadsat interpose at 5 g/100ml,

Figure 3. Plates (A-O) showing the genotoxic effects of seed priming of *Punica granatum* peel aqueous extract (PGPAE) in germinating root tips of *Lathyrus sativus* L

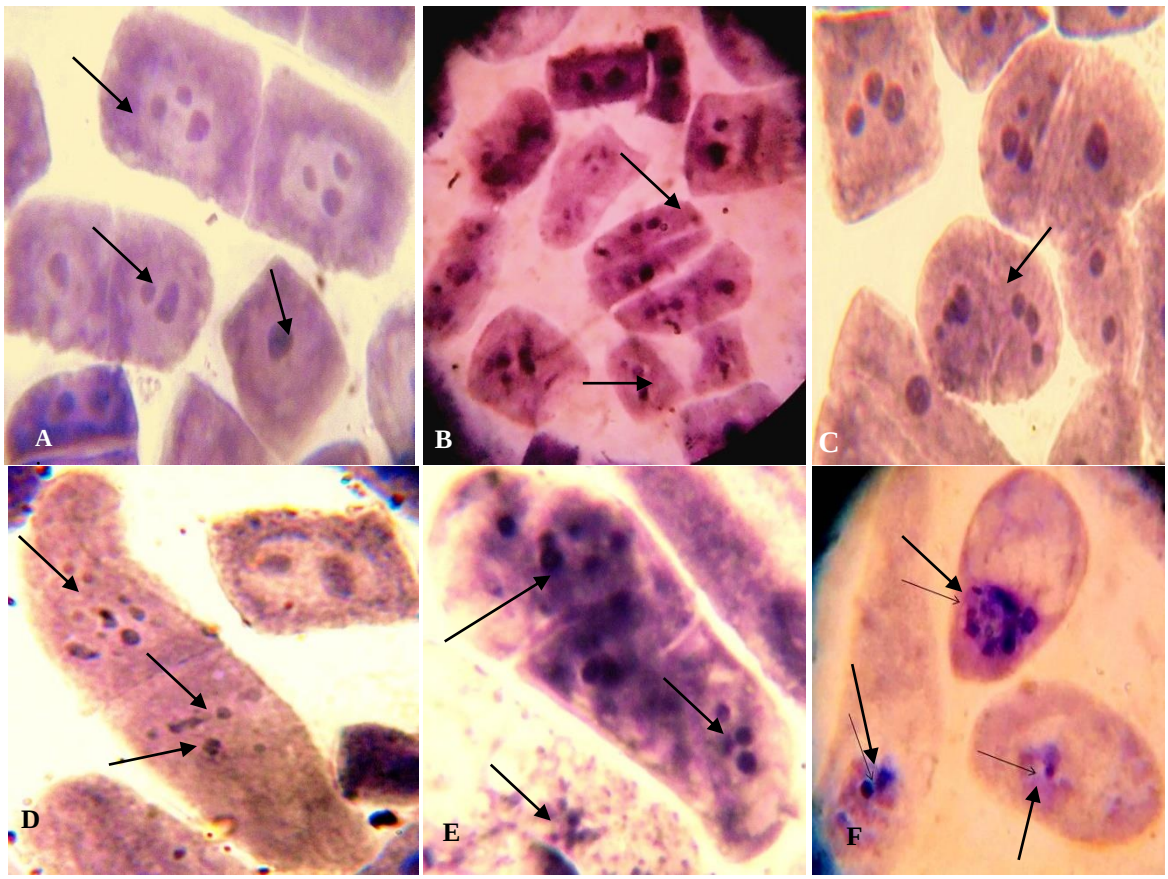


G: Giant diad cell with vacuolated prophase and quasi-sate metaphaseat 5 g/100ml,
H: micronuclei, fragments at ball anaphase at 7.5 g/100 ml,
I: giant strap cells with karyorrhexis and micronuclei at 7.5 g/100ml;
J: Interphasic nucleus with cytoplasmic shrinkage at 10g/100 ml;
K: necrosis withprotoplasmic vacuoles, eroded ball metaphaseand chromosomal fragmentations at anaphase with dislodged equatorial axis.
L: apoptotic cells having fragmentated nucleochromatin materials lodged at corners with cytoplasmic erosion at 10 g/100 ml;
M: nuclear degradation leading to apoptosis in distorted cells in giant cells at 10 g/100 ml,
N: Giant cell with nuclear fragmentation with nuclear blobs asapoptotic responses;
O: Total Cell wall dissolution and protoplasmic shrinkage with degradedmulti-fragmentedkaryolysis at 10-12.5 g/100ml leading to cellular death.

Fig 3. Plates (A-O) showing the genotoxic effects of seed priming of *Punica granatum* peel aqueous extract (PGPAE) in germinating root tips of *Lathyrus sativus* L. (continued)

3.7. Acute cytotoxicity measurement

The effect of PGPAE in germinating root tip Nucleolus frequency, dynamics and volume and Nucleolar Index in root tip cells using Hematoxylin staining is presented in the figures 4-5.



A: Mono, di and tri-nucleolated cells (0.625 g/100 ml) ,
B: Diminishing nucleolar volume with fragmentation (1.25 g/100ml),
C: Diminishing nucleolar volume with nuclear fragmentation 2.5 g/100ml,
D: Diminishing nucleolar volume with cytoplasmic shrinkage (5 g/100ml);
E: Micronuclei clusters with diminishing volume with cytoplasmic shrinkage (7.5 g/100 ml);
F: Nucleolar fragmentation fragmented nucleoli clusters in apoptotic strap cells (10 g/100ml)

Figure 4. Plates (A-F) showing the nucleolar degradation (acute cytotoxicity) effects after seed priming of *Punica granatum* peel aqueous extract (PGPAE) in germinating root tips of *Lathyrus sativus* L., variety Bidhan Khesari

The findings after the pre-treatment experiments using *Punica granatum* peel aqueous extract (PGPAE) proved that the pomegranate epicarp aqueous extract was cytotoxic at the concentrations (1.25 to 10 g/100 ml). The changes observed in the meristematic cells of *Lathyrus sativus* L. subjected to pre-treatments with the extract at the concentrations of 0.625 to 7.5 g/100 ml were shown in Fig. 3 with various photomicrographs. Thus, these findings suggested that in addition to its significant genotoxic action, the extract may also have extroversive end-point cytotoxic properties through disruption of normal nucleolar dynamics in root tip cells during germination. Among the chromosomal anomalies c-metaphases and other clastogenic changes were prominent. In addition, the plant extract-treated cells displayed cytoplasmic vacuoles, which cleared protoplasm at 5 and 7.5 g/100 ml pre-treatment sets of root tips, and these cytoplasmic vacuoles gradually produced clearing of protoplasm with a fragmented nucleus pushed towards the corners of the cell periphery—a feature not initially observed in the cells treated with lower concentrations. Phase indices were greatly altered in the roots subjected to the aqueous extract at concentrations of 1.25 to 7.5 g/100 ml,

suggesting a cytotoxic effect. As illustrated in figure plate 1, in contrast to lower treatments, a significantly larger proportion of cells undergoing apoptosis and exhibiting nuclear alterations cum fragmentations with apoptotic blobs associated with cell death processes. Apart from the cytoplasmic vacuoles, in this instance as well, the proportion of cells exhibiting normal cell divisional phases were significantly decreased. These findings could prove that *Punica granatum* peel aqueous extract (PGPAE) had produced significant cytotoxic as well as genotoxic effects at this highest dose. The overall changes in the phase indices with statistically significance values were indicative of this claim. In addition nucleolar index (NI) (Fig. 3) which is a "cytotoxic end-point bio-marker" of stress tolerance had also been observed after germination within pre-treated root tips of the afore-mentioned doses. The normal nucleolar frequency and volume got significantly altered with gradually increasing doses of PGPAE pre-treatment in the germinating root tips of *L. sativus*, worth to mention which was indicative of dose-dependency that was inversely proportionate in manner. With gradually increasing doses of pre-treatment there were increase in the number of actively dividing nucleoli (in 1.25 and 2.5 g/100 ml pre-treated root tips), but the volume of the nucleoli could be found to be diminishing as compared to the lowest dose pre-treated root tip squashes. Increasing number of nucleoli was found to accompany with gradually shrinking protoplasmic volume, clearing of cytoplasm, nuclear fragmentations and formation of giant strap cells with micronucleoli with multiple fragmentations in 5 and 7.5 g/100 ml pre-treatment sets (Fig. 4). Significant statistical correlations were drawn which demonstrated the changes in the nucleolar dynamics (frequency) in comparison to control in all pre-treatment doses further confirming the cytogenotoxic potential of *Punica granatum* peel aqueous extract (PGPAE) as a strong potential cytotoxic agent for germinating somatic cells (Fig. 5).

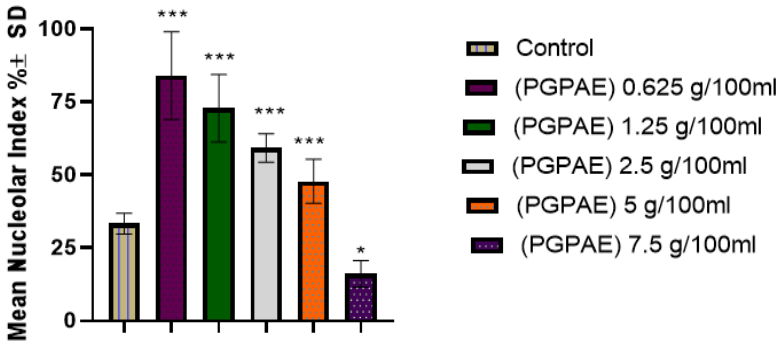


Figure 5. Abnormal Nucleolar index induced by *Punica granatum* peel aqueous extract (PGPAE) pretreatment in germinating root tip cells of *Lathyrus sativus* L. Different asterixes within in a column represent significant difference at $p < 0.05$ after analysis of one way ANOVA in comparison to respective control followed by Brown-Forsythe and Welch's Post-hoc test to reveal the level of significance of mean each group in comparison to control.

3.8. Antioxidant assaysemploying PGPAE

The 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging capability of PGPAE is presented in the figure 6. Effect of scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) by *Punica granatum* peel aqueous extract (PGPAE) was investigated. It was commonly known that the activity of scavenging proton radicals was one of the various antioxidation processes. One of the substances with a proton free radical had been DPPH, which exhibited a distinctive purple absorption at 517 nm [46, 47]. The purple colour of DPPH would quickly disappear when it came into contact with proton radical scavengers [48]. *Punica granatum* peel aqueous extract (PGPAE) fraction had been found to possess superior scavenging capacity on DPPH radicals at a dosage of 200 µg/mL (95.65 ± 3.14 %) [Each result indicates mean ± standard deviation (n = 3)]. This was seen in comparison to the control BHA (97.98 ± 4.21 %). PGPAE would prove to be a great antioxidant reservoir since scavenging effect could be accounted even at lower concentrations (25 and 50 µ/mL) which were nearly above 50% (Fig. 6).

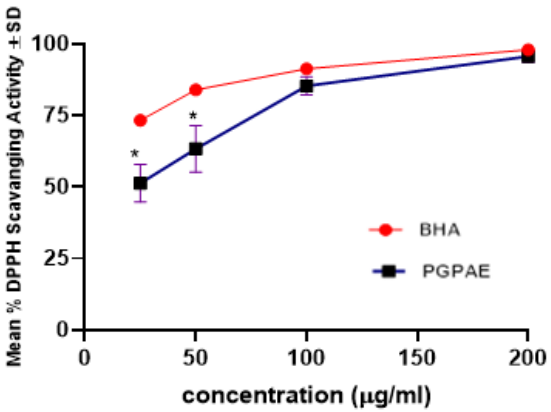


Figure 6 In this study, several *Punica granatum* peel aqueous extract (PGPAE) concentrations were tested for their ability to scavenge DPPH radicals at concentrations between 25 and 200 µg/ml, using a standard BHA = Butylated Hydroxy Anisole. The figures showed the mean (n = 3) plus standard deviation. Every experiment was conducted thrice, and the average ± standard deviation for each sample's three replicates was displayed. The statistically significant difference ($p < 0.05$) was ascertained using paired "t" test at each concentration levels (BHA vs PGPAE; t=2.540, df=3; Correlation coefficient (r)=0.9817, P value = *<0.0091, one tailed).

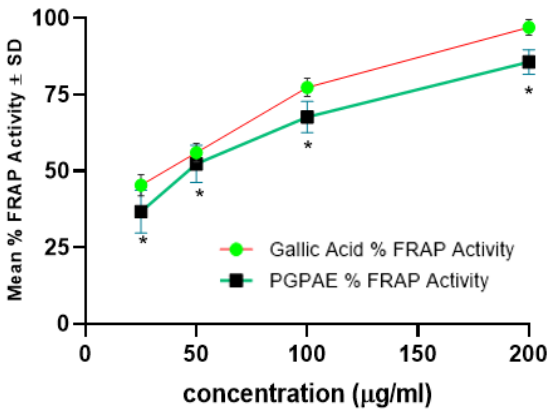


Figure 7 Scavenging effect of different concentrations of *Punica granatum* peel aqueous extract (PGPAE) in FRAP assay in comparison to Gallic acid as standard antioxidant. Every experiment was conducted thrice, and the results were displayed as the mean ± SD for each sample's three replications. The statistically significant difference ($p < 0.05$) was ascertained using paired "t" test at each concentration levels (GA vs PGPAE; t=5.051, df=3; Correlation coefficient (r)=0.9928, P value = *<0.0036, one tailed).

Ferric ion reducing / antioxidant power (FRAP) of PGPAE is presented in the figure 7. The antioxidant capacity of foods and biological samples had been assessed using a wide range of techniques in recent years [49]. Thus, the FRAP assay was used to assess the total antioxidant potential of *Punica granatum* peel aqueous extract (PGPAE). This assay in which a ferric salt Fe(III) (TPTZ)2Cl3 (TPTZ = 2,4,6-tripyridyl-s-triazine) was used as oxidant [38] took the advantage of electron transfer reactions [49]. Regarding antioxidant capacity of the *Punica granatum* peel aqueous extract (PGPAE) in the present study, it was clear that PGPAE (ranging from 50-200 µg/ml), had been able to perform >50 % FRAP activity at 50 µg/ml followed by 100 and 200 µg/ml concentrations up to a level of 90% of reducing power at 4 and 30 min (125 and 165 mol Fe(II) per gm sample dry weight, respectively). This positive correlation with Gallic acid as standard would prove the superior Fe (III) reducing power of PGPAE (Fig. 7).

3.9. Anti-haemolytic (against H_2O_2 induced ROS) and anti-tissue damaging activity of PGPAE

The effect of PGPAE in chicken RBCs is presented in figure 8.

From the histogram it could be found out that chicken 2% RBC when exposed to PGPAE after H_2O_2 treatment, got partial to prominent protection against H_2O_2 induced free radical induced membrane damage. PGPAE at 50, 100 250 µg/ml concentration was found to be weak, showing inhibition of free radical induced haemolysis % slightly >20% than of the positive control, giving only 20.09%, 23.75% and 29.83% inhibition respectively; however at 500, 1000, 2000 and 4000 µg/ml concentrations. PGPAE could significantly diminish the free radical generated membrane damage showing potent activity, with inhibition of haemolysis % (<80%) than that of the positive control, i.e., 52.4%, 65.29%, 71.82% and 89.48% inhibition respectively. Therefore it becomes clear that 500, 1000, 2000 and 4000 µg/ml concentration of PGPAE would have successfully been proven to be anti-haemolytic in nature *in vitro* 2% chicken RBCs can act as a significant cytoprotectant *in vitro* (Fig. 8).

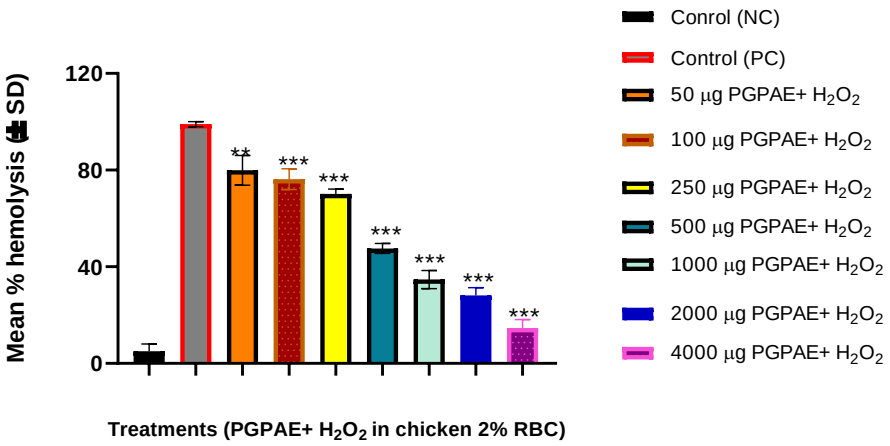


Figure 8. The results were expressed as Mean% haemolysis ± SD, (n=5) in H_2O_2 treated chicken 2% RBC followed by PGPAE treatment. Every experiment was conducted thrice, and the results were displayed as the mean ± SD for each sample's three replications. The statistically significant difference was ascertained by Brown-Forsythe ANOVA test: F= 425.0, d.fd= 20.95, p <0.001),**p<0.008, ***p<0.001 followed by post-hoc Dunnett's T3 multiple comparison test (vs. Positive control, PC).

The effect of PGPAE on Human RBCs is presented in the figure 9. From the histogram it could be found out that chicken 2% RBC when exposed to PGPAE after H_2O_2 treatment, got partial to significant, i.e., above 80% protection against H_2O_2 induced free radical induced membrane damage. PGPAE at 50, 100, 250 and 500 µg/ml concentration was found to be weak, showing inhibition of free radical induced haemolysis % slightly > 20% than of the positive control, giving only 19.79%, 24.8%, 31.5% and 45.02% inhibition respectively; however at 1000, 2000 and 4000 µg/ml concentration, PGPAE could significantly diminish the free radical generated membrane damage showing potent activity, with inhibition of haemolysis % <80% than that of the positive control, i.e., 56.75%, 69.75%, and 79.48% inhibition respectively. Therefore it become clear that 1000, 2000 and 4000 µg/ml concentration of PGPAE would have been successfully proven to be anti-haemolytic in nature *in vitro* 2% Human RBCs showing superior cyto-protection *in vitro* (Fig. 9).

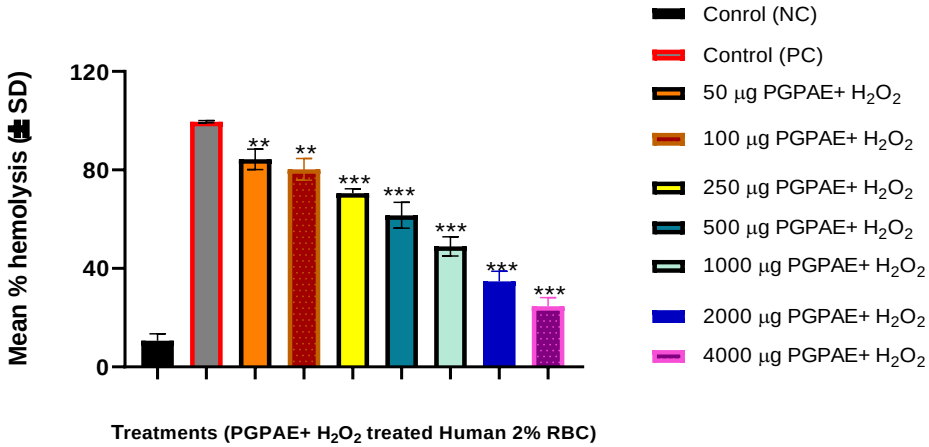


Figure 9. The results were expressed as Mean% haemolysis ± SD, (n=5) in H_2O_2 treated human 2% RBC followed by PGPAE treatment. Every experiment was conducted thrice, and the results were displayed as the mean ± SD for each sample's three replications. The statistically significant difference was ascertained byBrown-Forsythe ANOVA test: F= 334.8, d.fd= 26.29, p <0.001), **p<0.005), **p<0.002, ***p<0.001 followed by post-hoc Dunnett's T3 multiple comparison test (vs. PC; positive control).

Anti-tissue damaging activity of PGPAE revealed that among three tissue types PGPAE could significantly inhibit the tissue damaging activity against Triton X treated positive controls. All the sets were found to undergo anti-haemoglobin releasing activity (*in vitro*) and in case of chicken liver tissues PGPAE could inhibit maximum release of haemoglobin (34.09±4.07% in comparison to 58.39±4.186% positive control) followed by chicken lung tissues (36.51±3.44% in comparison to 47.98±3.93% positive control). There were least protection during haemoglobin release from chicken heart tissues (33.28±4.189% in comparison to 38.17±1.506% positive control) by PGPAE. It thus concluded that PGPAE has a strong tissue protection activity *in vitro* (Fig. 10).

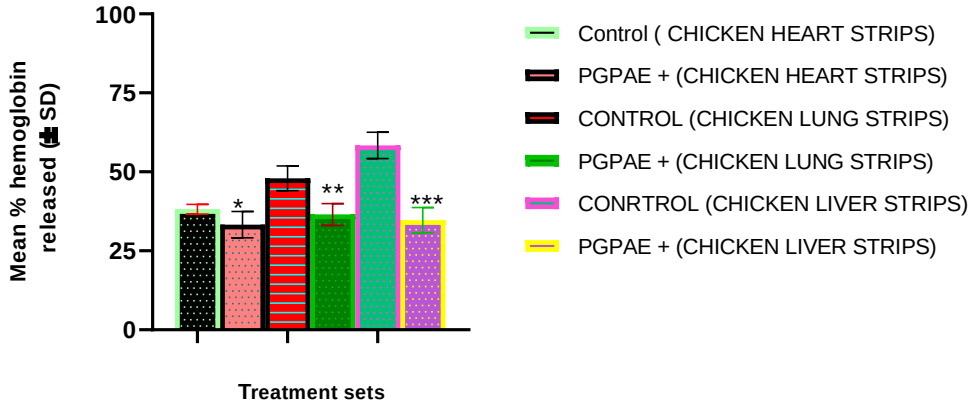


Figure 10. The results were expressed as Mean% haemolysis ± SD, (n=5) in chicken tissue strips followed by PGPAE treatment vs Triton X treated positive control sets. One tailed "t" value of each test sets were as follows: (F= 3.007df= 4,*p<0.0198; F= 14.08 df= 3, ***p<0.0004; F= 4.731 df= 4, **p<0.0045 respectively).

4. Discussion

The most significant challenge for health care professionals could be to explore different plant extracts that had been already in use, with antimicrobial properties vis-a-vis their underlying mechanisms of actions. Equally daunting could be exploring the structural components that would have been responsible for such activities and for the identification of altruistic safer doses using these extracts or components as comprehensive tools for the prevention of microbial contaminations in pathological invasions. Despite the vast number preliminary studies that had been conducted in this area, research had gained essential momentum in the past few years [50]. Even though phytochemicals could possess moderate to strong antimicrobial activity, very few medicinal and cultivated plants till today have had been thoroughly examined for their potential as safe phytochemical extracts for human consumption in addition to their antimicrobial activity. Regular human activities led to the use of antibiotics causing microbes to evolve rather in faster rates to achieve varying degrees of resistance.

Additionally, this nuisance caused undesirable side effects and the production of expensive synthetic medications [51]. In the wake of this peril, an effort had been initiated to expand on the notion of microorganism's sensitivity and to switch the emphasis to plant-derived compounds as a medication to kill harmful and pathogenic bacteria. In the present study, PGPAE had been the reservoir of various classes of phytochemicals, such as flavonoids, alkaloids, phenols, glycosides, and steroids that have been experimentally tabulated. According to a qualitative examination of phytochemicals, the phytochemicals that had been separated from the pomegranate fruit peel extract included phenols, tannins, proteins, and carbohydrates. The results of this qualitative and quantitative analysis (Table: 1 and Table: 2) closely resemble those of previous research. A number of separate classes of pomegranate fruit crude organic extracts had also shown impressive efficacy against particular microbiological strains. Antimicrobial activities of the phytochemicals of PGPAE had been compared with the sensitivity of the common antibiotics, penicillin and amoxicillin available.

The analysis of the aforementioned data

effectively demonstrated how the findings align with publications demonstrating the antibacterial effectiveness of various medicinal plants in treating microbiological illnesses [52]. In contrast to some previous studies, some of the medicinal plants showed insignificant activity against the different bacterial strains [53] owing to the method of extraction of the plant extracts and different phytochemicals present within the extracts/decoctions [54]. Some of the levels of close similarities and varying levels of differences in respect to the measure of sensitivity of bacterial strains might be due to the antimicrobial properties of plant extract and the resistance mechanism of the microorganism *in vitro*. Significant inhibition with silk fibroin-chitosan blend ZnO NPs to the bacterial strains and remarkable inhibition zones were measured due to antibacterial activity following agar well diffusion method [42]. Similar type of experiments were also performed using TiO_2-NPs and ZnO-NPs to show the antibacterial potentiality against different bacterial strains [55]. Sometimes same extract/decoction prepared after treatment with same strains of bacteria could show varying levels of sensitivity out of seasonal variations during fruit setting, ripening and storage that might affect phytochemical compositions responsible for antimicrobial activities *in vitro* [56]. Plants produced the true bioactive phytochemicals along with the several chemicals during its growth, which modulate human physiology and also determine the true medicinal value to the phytochemicals per se [57].

Environmental variations augment stresses in plants and plants in turn produce and release of secondary metabolites like phenolic compounds, flavonoids, flavanols, Glycosides, alkaloids and polyacetylene that inhibit microbial growth and show lethal effects against microorganisms [58, 59]. These substances confirmed slight toxicity in host cells, thus could be used in new antimicrobial drug development [60]. Strong inhibitors, such as flavonoids and phenolic compounds, had been found in the peel of pomegranates, according to phytochemical research [61]. It was well documented that certain polyphenolic compounds might inhibit microbial growth by in combination with one another. Through the formation of complexes with soluble and extracellular proteins found in microbial cell walls, these compounds provide driving forces that inhibit infection. These complexes precipitate membrane proteins and inhibit enzymes like glycosyl transferases, leading to the disintegration of the microorganisms [62, 63]. Pomegranate peel extract has a potent antibacterial impact on all bacteria, including *Salmonella* and Gram-positive *S. aureus*. This was supported by the significant levels of tannins in pomegranate peel extract shown to be particularly effective against some strains of the *Staphylococcus aureus* [3]. Recently, pomegranate peel's antibacterial efficacy against the plant disease *Ralstonia solanacearum* was assessed *in vitro* [64]. When pomegranate peel was added to bacterial cultures at varied dosages (5, 10, and 15 mg/mL), the bacterial cultures displayed a more gradual deregulated survival rate in comparison to the control. The bacteria that were treated had their cytoplasmic membrane and cell wall detached, which dramatically decreased the organism's motility. In this study, the group receiving 5 mg/mL of pomegranate peel extract had the least diameter. It's interesting to note that the current study supported the facts of earlier research showing that concentrations of PGPAE of 10 mg/ml and 5 mg/ml were efficient in exhibiting antibacterial effects against *Escherichia coli*, *Bacillus sp.*, *Klebsiella sp.*, and *Priestia* sp., (Table: 4; Fig. 2).Additionally, two triterpenes extracted from pomegranate peel—ursolic acid and Asiatic acid—augmented significant antimicrobial activity against *E. coli, S. aureus*, and *M. smegmatis*, according to a study by Sun *et al.* (2021) [65] that examined the antimicrobial activity of phenolic compounds from pomegranate peel. When the integrity of the cell membrane was broken, punicalagin was able to connect to the functional domains of the bacterial transcriptional regulator PhcA. This resulted in a disordered regulatory network and eventually bacterial impairment. According to reports, pomegranate's antibacterial mechanism involved membrane protein precipitation, which caused microbial cell lysis [66]. Recent research had demonstrated the antibacterial efficacy of eight distinct pomegranate peel ethanol extracts against *S. aureus* [67].

Peroxidases (EC number 1.11.1.x) are an

important group of enzymes which catalyze many oxidative reactions to overcome the toxic effect of hydrogen peroxide or organic peroxide and play a vital role in various metabolic processes in plant tissues. The enzyme polyphenol oxidases (EC number 1.10.3.x) are a group of Cu-containing enzymes which can able to oxidize polyphenols found in fruits and vegetables, causing post-harvest disorders along with many other functions. The presence of oxido-reductive enzymes in the mature fruit peel might help (Table: 3) the fruit as well as the seeds from the toxic effect of the peroxide and other free radical moieties during pathogenic infection. The antimicrobial property of the crude extract may also explain the presence of oxidoreductive enzymes in the fruit peel. This means that rather than being thrown out as leftovers, the crude or fresh pomegranate peels could be further utilized to purify these peroxidases and polyphenol oxidases to prevent antimicrobial contamination within food products [68] and sprouts preservations for longer shelf life.

Flavonoids are present in a large range of fruits, leaves, and flowers and constitute an important source of nutraceuticals in human diets. What distinguishes them are either their two benzene rings (rings A and B) connected by a 3-carbon bridge (creating chalcones) or their pyrene or pyrone ring (ring C). Based on the locations of the A, B, and C rings and the modifications made to them, the more than 4,000 flavonoids, discovered so far, could be categorized into several groups. Flavanols, flavones, isoflavones, and anthocyanin pigments happened to be some of these classes. While the production of red and purple anthocyanin pigments has been the most obvious function of flavonoids, certain non-pigmented flavonoid compounds also had been implied to play important biological roles in plants [61], which being allelochemicals prevented root growth and apoptosis [69]. In addition to the strong antioxidant qualities exhibited by many flavonoids and phenolic compounds [70], a recent study revealed [71] that there might be a positive correlation between the cell's REDOX potential and the regulation of root death caused by mitochondrial apoptosis-signalling following suboptimal accumulation of these flavonoids within the living cells. The potent phytotoxin flavonoid (-)-catechin had also been found in *Punica granatum* L. peels [72]. This allelochemical in root cells triggered reactive oxygen species (ROS) signal transduction pathways including Ca^{2+} translocations, which might have activated in the roots of sensitive plants upon exposure to (-)-catechin and ultimately leading to cellular apoptosis. This study discovered that seed priming PGPAE (for 24 hours) at concentrations ranging from 0.625 to 7.5 g/100ml in the germinating root tips of *Lathyrus sativus* L. could cause the early onset of chromosomal aberrations such as pole to pole metaphase stickiness and clumping, tropokinesis, dislocated fragments, polyploidy with coagulated anaphase chromosomes, giant cells multi-vacuolated somatic diads, giant cells with quasi-state metaphase, micronuclei, fragments at ball anaphase, giant strap cells with karyorrhexis and micronuclei etc. (Fig. 3) in a dose-dependent manner which are in harmony with earlier reports [73, 74].

Cellular checkpoints aid in ensuring the proper sequence of events to occur following environmental shocks such as spindle or DNA damage. In this reaction to damage further, this mechanism might halted the cell cycle, giving the cells time to fix damaged DNA or finish assembling spindles before the cycle continues [75]. However, the cell cycle regulatory system responded immediately to imperfect DNA replication or DNA damage by either preventing or delaying entry into mitosis. At DNA damage checkpoints, this regulation produced a negative signal to a sequence of protein kinases, which halted the cycle. PGPAE, which is made up of a "cocktail-of-differential-allelochemicals," might thereby lowered the mitotic indices, allowing the cells to adjust to the G_2 checkpoint (DNA Damage Checkpoint). The uncompromising activity of cyclin-dependent kinases could be brought forth by changes in kinase activities, specifically phosphorylation and dephosphorylation, preventing mitosis. Pomegranate peel has the largest quantities of high molecular weight phenolic chemicals, such as polyphenols, which have the potential to cause pleotropic biological effects at the cellular microcosm, particularly nuclear toxicity, in addition to being antioxidants. The ability of *Punica* polyphenols, in this aqueous extract, to prevent and impede the metaphase to anaphase transition suggested that tubulin

subunits were the main targets as per earlier studies. This explanation was consistent with earlier studies conducted by Sorger *et al.* (1997) [76]. They found that the majority of medications causing microtubule damage led cells to stop at the metaphase-to-anaphase transition because of the dysfunctional spindle-attachment checkpoints. By de-polymerizing microtubules or by boosting microtubules to destabilise, these substances prevented proper spindle formation. Ever since sister chromatid separation had been prevented, the mitotic spindle's disintegration produced a strong signal that lengthened the metaphase phase, and it could even cause all chromosomes to segregate toward the extreme poles [77]. Failure of the cytokinesis process owed to a deficiency in tubulin equilibrium caused by the polymerization and de-polymerization of microtubules, as their expansion would be necessary for the production of phragmoplasts by new microtubules [78]. Several publications had previously reported the observation of mitotic arrest during metaphase stage due to alterations in the mitotic spindle [74, 79, 80]. It had been evident that the water extracts from *Rosmarinus offisinales* and the polyphenol extract from *Punica* [81] slowed downfall of the cell cycle. Initially, the majority of cells at the G_2-check point were blocked by both extracts; subsequently, they obstructed the cells that crept into at the metaphase check point. Moreover, various flavonoids such as quercetin, rutin, and kaempferol, as well as phenolic acids containing tannins (catechin), such as chlorogenic acid, caffeic acid, and gallic acid, had been found to have antiproliferative properties in plant cells at variable doses [80]. Furthermore, during the metaphase-to-anaphase transition, the chromosomes disaggregated due to malfunctions in their regulatory mechanisms. Osmolytic imbalance and increasing turgidity in the growing root cells might thus resulted out of a notable accumulation of PGPAE's phenolic compounds and high molecular weight flavonoids [82]. Cells that were unable to carry out checkpoint adaptation must instead have started a program of cell death or simply fail to proliferate, remaining quiescent in mitosis, depending on the kind and degree of cell injury as well as the level of stress [83]. Large vacuole formations and membrane disruption caused by saponins and tannins in the aqueous extract would have changed the protoplasm's turgidity and sparked apoptotic and necrotic reactions that resulted in the formation of enormous ghost cells (Fig. 3). The last stage was the collapsing of tonoplast and the release of hydrolytic enzymes (also known as vacuolar processing enzymes) into the cytoplasm, which quickly broke down the entire protoplast (Fig. 3). Only empty cells with cell walls were left after PCD set in [84, 85]. The cumulative sequential effects of the allelochemical cocktail found in PGPAE, which was extremely cytotoxic to *Lathyrus sativus* L. root tip cells in germination state, was responsible for all these PCD reactions. Same responses had been observed in *Allium sativum* and *Lathyrus sativus* L., root tips after pretreatment with seed aqueous extract of *Cascabella thevetia* L., [35] rich in alkaloids and allelochemicals. There would have been a positive correlation between ROS outburst owing to disorganized cellular homeostasis and ROS-mediated localized and long-distance signalling leading [86] to root necrosis / apoptosis within germinating root tip cells after pre-treatment with PGPAE's phenolic compounds and high molecular weight flavonoids.

The late prophase stage of mitotic division takes place when nuclear cells disintegrate frequently. In this experimental setup (Fig. 4), the typical hallmark features of dead cells [87] could be seen, including plasmolysis, cell membrane breakage, vacuole collapse, and cytoplasm degradation with shrinkage away from the cell wall (Fig. 4). The nucleolar poisoning effects of PGPAE, which disrupted the nuclear membranes at 5, 7.5, and 10 g/100ml seed priming in the germinating root tip cells of *Lathyrus sativus* L., could be explained by the significant changes in nucleolar dynamics and frequency (Fig. 4, 5). This total collapse of nucleolar architecture ultimately disrupted the entire pool of r-DNA [32]. The studied medicinal plant's whole polyphenol extract, *Punica granatum* L., was able to alter the meristematic cells of *Allium cepa* L.'s ultra structure and enhance the quantity and size of the vacuolar system [81]. The increased quantity and size of vacuolar systems, many of which seemed to include cellular detritus in varying states of degeneration, had been proposed by Fuzinatto *et al.* (2007) [88] as an outcome of the

preponderance of autophagic and autolysis mechanisms. Certain types of plant programmed cell death, hypoxic stress [89], and oxidative stress (ROS production) in BY-2 tobacco cells had all been linked to alterations in nuclear morphology [90]. Madeo *et al.* (2002) [91] showed that the appearance of randomly distributed nuclear fragments in the cytoplasm of yeast cells and the induction of disintegrated nuclear envelope were similar to the final stages of PCD and also similar in the nucleolus of the root tip cell of *Lathyrus sativus* L (Fig. 4) following PGPAE seed priming in the present experimental set up. Beers and McDowell (2001) [92] stated that one of the characteristics that could distinguish plant cells with programmed cell death had been the separation of the plasma membrane from the cell wall. This phenomenon had also been accounted concurrently with PGPAE seed priming in the root tips of *Lathyrus sativus* L. (Fig. 3, 4). Furthermore, Schussler and David (2002) [93] demonstrated that early in plant programmed cell death, a change in the position or structure of the plasma membrane had taken place. Furthermore, as the cell death pathway progressed, this modification of the plasma membrane was gradually seen in soybean cells [94]. According to Tawab *et al*. (2004) [81], the process of nuclear structural alterations, cytoplasmic condensation, and cytoplasmic integrity collapsed in the periphery of the cell until the late stages of the death process were systematically sequential and must be attributed to genetically-controlled cell death in plant cells. The ultimate hallmark of genotoxicity [32, 95] could be the disruption of the nuclear membrane with complete disintegration of the nucleolar architectural dynamics in relation to the nucleolar volume with concomitant release of nucleoprotein complexes in the cytoplasm. Thus the decisive predisposition of cellular genotoxicity coupled with chromosomal aberrations [96] had been prominently brought forth by PGPAE in *Lathyrus sativus* L. root tip cells. Therefore, it might be conclusively opined that the cytotoxic dose range for *Lathyrus sativus* L., for genomic disintegration and genotoxicity has been in the range of 7.5-10 g/100ml of PGPAE. Secondary metabolites produced by plants also called allelochemicals have been able to inhibit germination. The inhibition depends on the plant species type, its origin and extracts concentration [97]. Several plant substances with identified allelochemicals had been reported to have phytotoxic property to kill weeds and could be exploited as natural herbicides [98]. The allelopathic effects of these phytochemicals resulted in their use as bio-herbicides to control weeds in agriculture [99]. Phenolic compounds and their degradation products had phytotoxic activity causing inhibition of seed sprouting and root development disruption. Exploitation of phenolic allelochemicals has to be done to use them in weed management [98]. In order to identify the active ingredients in the pomegranate peel extract, a qualitative analysis was conducted on the aqueous extract in the current experimental setup. The results clearly demonstrated that PGPAE should be carefully utilized as a potential bio-herbicide.

PGPAE was added (*in vitro*) with varying doses with DPPH in order to assess the free radical scavenging activity. A stable free radical with a purple colour and an absorption band at 517 nm is called DPPH. By absorbing an electron or hydrogen radical from an antioxidant, it is reduced to 2, 2-diphenyl-1-picrylhydrazine (yellow in colour) [99]. According to Hseu *et al.* (2008) [100], this radical is sensitive enough to identify even minute amounts of antioxidant chemicals found in various extracts. Pomegranate peel aqueous extract shows a notable dose-dependency in its inhibitory action against the DPPH radical in the current investigation (Fig. 5). Previous research had showed that water, an environmentally benign solvent, worked very well for extracting antioxidants from pomegranate peels [101, 102, 103].

The FRAP assay [38] is another frequently employed technique. Based on the reaction principle outlined in the materials and techniques section, it can be utilized to quantify the antioxidants' overall total reducing power. For the reasons explained, the FRAP assay was selected to evaluate the antioxidant characteristics of fruit peel, pulp, and seed fractions. With the FRAP assay, the antioxidants in the samples were first handled as reductants in a redox-linked colorimetric reaction. Secondly, the technique for the FRAP assay has been highly simple and may be easily standardized. However, we do believe that the FRAP assay is still appropriate for determining the fruit samples'

antioxidant activity. The antioxidant activity of most fruit peels was 25% higher than that of fruit pulps, based on data from the different studies. The origin of this observation was fully explained by the difference in water content between the peel and pulp parts. Using the FRAP assay, Halvorsen *et al.* (2002) [104] determined the total antioxidant concentrations of a few fruits. We attempted to create a relationship between the fruit FRAP values found during this investigation and the data from other sources. Based on seasonal variations, we determined that the peel fractions of pomegranates were only marginally less moist than the pulp fractions after measuring the water content of the pulp and peel fractions (data not shown). Therefore, as fruit peel fractions have the potential to be important sources of antioxidants, more research should be done on them, particularly with those having very high antioxidant activity. In comparison to seed extracts, pomegranate peel extracts demonstrated higher levels of antioxidant activity *in vitro*, according to Singh *et al.* (2002) [101]. According to a subsequent study conducted in the same laboratory, Pomegranate peel extracts might thus significantly protect the liver against CCl_4 toxicity due to ROS damages [101]. The structural complexity and functional groups of different phytochemicals (polyphenols, alkaloids, flavonoids, tannins, saponins, terpenoids, glycosides, and total phenolic acids) vary greatly depending on the agroclimatic conditions and plant varieties, which further explained the results of these scavenging assays. This finding confirmed that the peels of the pomegranate fruit varieties under investigation showed significant antioxidant activity. Additionally, because of its strong natural antioxidant supply, peels might be used as a scientific foundation to support added value for medicinal and cosmetic formulations rather than being thrown away, which would further contribute to environmental pollution. *Punica granatum* fruit has been shown to have many beneficial properties, but there is still a dire need for more research on its pharmacological behaviour across a range of clinical applications and appropriate dosages in the treatment of human disorders. Particular attention should be paid to maximizing the fruit's benefits while minimizing any negative effects. Strong anti-haemolytic and anti-tissue-damaging properties were present in Pomegranate Peel Aqueous Extract (PGPAE) (Fig. 8, 9, 10). Biologically active compounds are produced by medicinal plants, some of which have unique flavours or tastes. It has been observed that several physiologically active compounds are beneficial as antioxidants and/or antibacterial agents. Antioxidants guard against oxidation and regulate the harmful effects of oxidation on biomolecules of living cells. Dietary antioxidants containing phenolic compounds are the most common kind of natural antioxidants [105]. These phenolic compounds function as singlet oxygen quenchers, hydrogen donors, reducing agents, free radical scavengers, and cell saviors, according to Fattouch *et al.* (2007) [106]. Nowadays, there is increased focus on the beneficial role that antioxidants play in preventing oxidative damage for the general health of human subjects worldwide [107]. Plant-based food products had been showing notable antioxidant activity because they contained high concentrations of phenols and polyphenolic chemicals [107]. By directly influencing the quantity of iron (III) ions in tissues, plant compounds such as flavonoids enhance quality of life [106]. Ebrahimzadeh *et al*. (2008) [108] claimed that flavonoids reduce issues connected to iron-oxidation by mobilizing tissue iron and excreting it in urine and faces. In this study, the absorbance reduction of a ferric-tripyridyl triazine complex to its coloured ferrous form rose from 25 to 200 µg/ml in a dose-dependent manner when antioxidants were present, especially in PGPAE. A significant metal ion stabilizing capacity was indicated by the extract's reduction in the concentration of the catalysis of the Fe^{+3}/Fe^{+2} transition in RBC during lipid peroxidation. When chicken and human washed red blood cells were haemolysed *in vitro*, *Punica granatum* fruit peel extract was also able to scavenge hydrogen peroxide in a concentration-dependent manner. Lipid peroxidation is a process that unsaturated fatty acids like arachidonic acid and linoleic acid go through [39]. Thus, the oxidation process involved the membrane lipids that included unsaturated fatty acids [109]. Unsaturated fatty acid oxidation once might be regulated by PGPAE. The free radical attack on erythrocytes was

caused by the oxygen transport and membrane concentration of polyunsaturated fatty acids (PUFA) associated with redox active haemoglobin molecules. Singlet oxygen and hydroxyl radicals (ROS) created from superoxide anion initiated lipid peroxidation [39, 40]. Thus, superoxide indirectly aids in lipid peroxidation [110]. Reactive oxygen species aid in the free radical assault that led to lipid peroxidation. *Punica granatum* fruit peel extract showed strong anti-haemolytic action in H_2O_2-induced haemolysis linked to membrane lipid peroxidation process system in both chicken and human washed red blood cells (*in vitro*) because of the extract's high total phenolic content. As a result, this aqueous decoction potentially must be employed as a nutraceutical, clinically to treat haemolytic disorders during chronic pathophysiological manifestations in experimental animals.

When it came to protecting the tissue strips against tissue disintegration, capillary rupture, and tissue haemoglobin release, PGPAE dramatically reduced the tissue damaging activity *in vitro* (Fig. 10). In a previous study, rats fed with pomegranate extract were shown to have significantly lower levels of MDA in their kidney, liver, and testis tissues observed after sacrifice. Pomegranates are also rich in punicalin, tannins, punicalagin, anthocyanins, gallic and ellagic acids, and vitamin C [103, 111]. Following previous studies [112] that adjusted the reduction of metal chelating proteins, matrix metalloproteases, and the natural antioxidant enzymes CAT, GPx, and SOD to stop the generation of free radicals to stop cellular damage or increase cell survival, PGPAE considerably prevented capillary tissue beds from rupturing and the simultaneous release of haemoglobin in the isolated liver, lung, and heart tissues in this study. Laboratory rats' kidney, liver, and testis all had considerably higher concentrations of SOD, GPx, and CAT when pomegranate extract was added [111]. Punicalagin isomers and anthocyanins, two phenolic chemicals found in pomegranates, have been likely to be responsible for all of these effects. These mixes are widely recognized for their ability to inhibit lipid oxidation and scavenge free radicals. The ease with which phenolic hydroxyl groups supply hydrogen for reducing agents is another finding reported by Madrigal-Carballo *et al.* (2009) [113] that indicates pomegranate polyphenolic compounds took part in redox reactions. Our results corroborated those of the earlier study [114], which found that groups receiving the polyphenol-rich pomegranate extract were able to maintain the structural integrity of liver and kidney tissues. It could be explained by pomegranate's antioxidant activity, which inhibited the body's natural production of free radicals and maintains ROS homeostasis while maintaining the integrity of the tissue microenvironment. Because pomegranates have antioxidant qualities, it is possible to deduce that pomegranate extract is more effective in raising the amount of antioxidant enzymes and lowering oxidative stress in various important organs in experimental animals like mice [115].

5. Conclusion

India has been among the largest producers of fruits worldwide. Pomegranate juices are extremely beneficial to recuperate deteriorating health hazards. But the peels are also the warehouse of different classes of allelochemicals and beneficial phytochemicals which must be judiciously explored and exploited as nutraceuticals, cosmeceuticals, pharmaceuticals and as biocides. Nonetheless, these different classes of beneficial phyto-constituents must be subjected to looking at and management as new drug molecules that might be clinically added with standard chemotherapeutic drugs to enhance their bioavailability in human subjects during clinical trials for better understanding of pathophysiological manifestations from early stages of prognosis during clinical trials. Additionally, these allelochemicals that are present in peels must be scientifically and judiciously explored as bio-weedicides and herbicides that would effectively replace their chemically synthesized counterparts which do augment severe ecological toxicity out of indiscriminate anthropological usage. Dietary consumption of aqueous decoction of pomegranate peel has been safe for cellular homeostasis and functioning of vital organs both in lower vertebrates (chicken) and human populace if effectively consumed during ailments to protect haematological health as a potent cyto-protectant against ROS outbursts. Rather than disposing out inadvertently in natural environment, this peel must be brought into well through-put employment

with different bio-assay guided isolation, purification and structure-function based bioassays for better understanding of the molecular interactions and mechanism of actions of different classes of phyto-constituents present herein, which have long been not put across for safer mass-scale human dietary consumption and practice. The varieties available in these climatic conditions might differ in its phytochemical constituents that affect the overall outcome showing partial shifting in its action on the chosen experimental models. Our results significantly matched with the previously reported claims as per record matched.

Declarations

Authorship credit and contribution statement

Authorship credit and contribution statement: Dr. Sisir Ghosh has performed the anti-microbial assays, draft writing, review and editing. Dr. Tuhin Ghosh has performed the FT-IR analysis and antioxidant assays, Dr. Dipan Adhikari has procured the samples and *Lathyrus sativus* seeds and performed all the phytochemical analysis and plant-cytotoxicity assay works and lastly Dr. Dipan Adhikari has performed the final conceptualization, Supervision writing with scientific review & editing, Validation of Methodology, statistical analysis and final format analysis.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the writing up, the authors used the Quillbot to improve the readability for enhancing the lucidity of the language. After getting the outcome authors reviewed and edited their individual part's content wherever needed and take full responsibility for the final content for publication.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding None.

Availability of data and materials

The data used to support the findings of this study are available from the corresponding author upon request.

Ethics approval and consent to participate

No human or laboratory animal was used in this research.

Consent for publication

Not applicable.

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