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Cytotoxicity and DNA Fragmentation Analysis of *Punica granatum* L. Peels Ethanolic (PPE) Extract against Human Gastric Cancer Cell Line (HGT-1)

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Abstract

Medicinal plants are considered a generous origin of antimicrobial compounds. *Punica granatum* commonly known as *Punica granatum* has developed as a medicinal plant with a possibility of anticancer activity. Un this present study investigated that the cytotoxicity and DNA fragmentation analysis of ethanolic extracts of *Punica granatum* peel using standard protocols. The strong FRAP (32.5 µM) and DPPH (28.6%) radical scavenging activity was noted in the 300µg/ml concentration of sample. The minimum cell viability (27.29%) and maximum cell inhibition were observed in the 500µg/ml concentration of the PPE extract. The inhibitory activity for the antiangiogenic effect of PPE extract with 65.50 ± 3.5% inhibitions was observed. The DNA fragmentation assay was performed for the confirming of cell death. The present study concluded that the *Punica granatum* peel ethanolic extract was observed the fragmentation of DNA and thus induces significant apoptotic activity.

***Key words*:** *Punica granatum,* Cell viability, Antiangiogenic effect, DNA fragmentation, FRAP

The plant derived extracts and essential oils are a potential source of natural and safer antibacterial, antioxidant, anticarcinogenic, antifungal, analgesic, insecticidal, anticoccidial and hypoglycemic agents [1-2]. During the growth, Plants generate a variety of secondary metabolites for their defense against negative biotic and abiotic environmental factors. Polyphenols, one of the main bioactive secondary metabolites, are natural antioxidant agents which have an important role in human health because of their ability to scavenge free radicals [3] which have been implicated in the development of a number of disorders, including cancer, neuro degeneration and inflammation [4-5] giving rise to studies of antioxidants for the prevention and treatment of diseases.

The foundation of modern cancer biology rests on a simple principle - virtually all mammalian cells share similar molecular networks that control cell proliferation, differentiation and cell death. The prevailing theory, which underpins research into the genesis and treatment of cancer, is that normal cells are transformed into cancers as a result of changes in these networks at the molecular, biochemical and cellular level and for each cell there is a finite number of ways this disruption can occur. Phenomenal advances in cancer research in the past 50 years have given us an insight into how cancer cells develop this autonomy. We now define cancer as a disease that involves changes or mutations in the cell genome. Current dogma states that cancer is a multi-gene, multi-step disease originating from a single abnormal cell (clonal origin) with an altered DNA sequence (mutation). Uncontrolled proliferation of these abnormal cells is followed by a second mutation leading to the mildly aberrant stage. Successive rounds of mutation and selective expansion of these cells results in the formation of a tumor mass. Subsequent rounds of mutation and expansion leads to tumor growth and progression which eventually breaks through the basal membrane barrier surrounding tissues and spreads to other parts of the body (metastasis). Death as a result of cancer is due to the invading, eroding and spread of tumors into normal tissues due to uncontrolled clonal expansion of these somatic cells.

Punica granatum (*Punica granatum* L.; Punicaceae) has gained popularity in recent years due to its multi functionality and nutritional benefit in the human diet. The fruit is rich in tannins and other biochemical, particularly phenolics, which have been reported to reduce disease risk [6-7]. *Punica granatum* fruit peel constitutes about 50% of the total fruit weight [8], and it is often discarded as waste. However, the fruit peel contains higher amounts of polyphenol compounds than the juice, and it possesses stronger biological activities [9]. Studies have shown that *Punica granatum* peel extract had markedly higher antioxidant capacity than juice extract in scavenging against superoxide anion, hydroxyl and peroxyl radicals and it inhibited $CuSO_4$-induced LDL oxidation [10]. Besides high antioxidant capacity, *Punica granatum* peel extracts have been reported to possess a wide range of biological actions including anti-cancer activity [11], antimicrobial activity [12], antidiarrheal activity [13], apoptotic and anti-genotoxic properties [14], anti-tyrosinase activity [15], anti-inflammatory and anti-diabetic activities

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[16]. Polyphenol compounds such as ellagic tannins, flavonols, anthocyanins, catechin, procyanidins, ellagic acid and gallic acid have been implicated in various pharmacological activities in the fruit peel [17]. However, the levels of these compounds in the *Punica granatum* peel may vary among *Punica granatum* cultivars which may result in differing levels of bioactivity [18].

In the production of *Punica granatum* juice, this is obtained by squeezing seeds, the *Punica granatum* peel is a waste product. Usually, after industrial processing, *Punica granatum* peel is used as an animal feed. Since *Punica granatum* peel has good nutritional and antioxidant properties, studies show that feeding cattle with *Punica granatum* peel significantly improves the nutrition of livestock and improves their health [19]. However, *Punica granatum* peel could have an even wider application. Namely, previous studies showed that, in comparison with other *Punica granatum* fruit parts, peel contains a high concentration of phenolic compounds, with hydrolysable tannins as main compounds [20]. For example, Alexandre *et al.*, proposed high pressure extracts from *Punica granatum* peel as a source of added-value biologically active compounds for application in food matrixes to increase antioxidant activity and to reduce the risk of pathogenic contamination [21]. The addition of *Punica granatum* peels extracts was successfully tested in yoghurt samples to increase its antioxidant content [22], in meat product to improve its oxidative stability [23] and in fruits to protect it from mycotoxigenic fungi [24]. Therefore, the aim of this study was to determine functional potential of *Punica granatum* peel ethanol extracts and to connect specific metabolites with the desired biological activity. The number of studies on tumours and multidrug-resistant bacteria has increased in recent years, and scientists are increasingly focusing on natural compounds that have a potential biological impact. Finding such compounds in biomass that is considered as a biological waste would be of great importance, mainly because large quantities of starting material would be easily reachable and in the same time, management of biological waste would be improved. Hence the present study aimed the antioxidant and cytotoxicity activities of *Punica granatum* peel extracts.

MATERIALS AND METHODS

Collection and preparation of Punica granatum peel extract

Fresh *Punica granatum* fruits were collected from a local market Thanjavur, Tamil Nadu, India. The fruit was thoroughly washed using distilled water and the seeds were subsequently separated to obtain the peel. The isolated peels were dried under shade at the room temperature for 3-5 days. The dried peels were ground into uniform coarse powder using a domestic blender. Approximately 200 g of *Punica granatum* peel powder upon extraction with ethanol (4–5 h, 90 °C) using a soxhlet apparatus yielded a bright red colour ethanolic *Punica granatum* peel extract (PPE). The extract was concentrated under reduced pressure (45–50 °C) using a rotary evaporator to obtain the viscous mass (42 g; 21.0% w/w). The PPE was stored at 4 °C in the refrigerator until further use in the experiment.

Antioxidant activity

DPPH scavenging activity

The free radical scavenging capability of *Punica granatum* peel ethanol extracts was determined through DPPH (1, 1-diphenyl-2-picrylhydrazyl) radicals [25]. Briefly, 4 mL of ethanol solution of DPPH (0.1mM) was mixed with 1 mL of methanol extract solution at different concentrations. The reaction mixture was incubated in a dark room for 30 minutes and the free radical scavenging ability was estimated by measuring the absorbance at 515 nm with the spectrophotometer. Ascorbic acid was used as positive control. The reaction was carried out in capped glass test tubes that were tightly wrapped with aluminum foil. The DPPH radical stock solution was freshly prepared every day for the reaction, and precautionary measures were taken to reduce the loss of free radical activity during the experiment.

FRAP

The ability to reduce ferric ions was measured using the method described by Benzie and Strain, [26]. The FRAP (Ferric Reducing Antioxidant Power Assay) reagent was generated by mixing 300 mM sodium acetate buffer (pH 3.6), 10.0 mM (tripyridyl triazine) TPTZ solution and 20.0 mM $FeCl_3.6H_2O$ solution in a ratio of 10:1:1 in volume. Samples at different concentrations (100,200,400,600 and 800 µg/ml) was then added to 3 ml of FRAP reagent and the reaction mixture was incubated at 37°C for 30 min. The increase in absorbance at 593 nm was measured. Fresh working solutions of $FeSO_4$ were used for calibration. The antioxidant capacity based on the ability to reduce ferric ions of sample was calculated from the linear calibration curve and expressed as mMol Fe^{+2} g^{-1}.

Reducing power activity

The sample together with Ascorbic acid solutions were spiked with 2.5ml of phosphate buffer (0.2 M, pH 6.6) and 2.5ml of 1% potassium ferricyanide. The mixture was kept in a 50°C water-bath for 20min. The resulting solution was cooled rapidly, spiked with 2.5ml of 10% trichloroacetic acid, and centrifuged at 3000rpm for 10 min. The supernatant (5ml) was mixed with 5ml of distilled water and 1ml of 0.1% ferric chloride and incubated for 10min. The absorbance was detected at 700nm on spectrophotometer. The extract concentration providing the absorbance was calculated from the graph of absorbance at 700 nm against extract concentration. Ascorbic acid was used as standard. Higher absorbance indicates higher reducing power [27].

Cytotoxicity activity

Cell culture

HGT-1 (Human Gastric cancer cells) cells were cultured at 37ºC under a humidified atmosphere of 5% CO_2, 95% air in Dulbecco's modified Eagle's medium supplemented with 20 mM sodium bicarbonate, 20 mM HEPES and 10% fetal calf serum without antibiotics.

MTT assay

The *Punica granatum* peel ethanol extracts were tested for *in vitro* cytotoxicity, using HGT-1 cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, the cultured HGT-1 cells were harvested by trypsinization, pooled in a 15 ml tube. Then, the cells were plated at a density of 1×10^5 cells/ml cells/well (200 µL) into 96-well tissue culture plate in DMEM medium containing 10% FCS solution for 24-48 hour at 37°C. The wells were washed with sterile PBS and treated with various concentrations of the PPE sample in a serum free DMEM medium. Each sample was replicated three times and the cells were incubated at 37°C in a humidified 5% CO_2 incubator for 24 h. After the incubation period, MTT (20 µL of 5 mg/ml) was added into each well and the cells incubated for another 2-4 h until purple precipitates were clearly visible under an inverted microscope. Finally, the medium together with MTT

(220 µL) were aspirated off the wells and washed with 1X PBS (200 µl). Furthermore, to dissolve formazan crystals, DMSO (100 µL) was added and the plate was shaken for 5 min. The absorbance for each well was measured at 570 nm using a micro plate reader (Thermo Fisher Scientific, USA) and the percentage cell viability and IC_{50} value was calculated using Graph Pad Prism 6.0 software (USA).

Anti angiogenic activity

Anti-angiogenic potential of drug samples was determined by CAM (Chorio allantoic membrane) assay [28]. The fertilized domestic chicken eggs were purchased from poultry trader Tanjavur, Tamil Nadu, were incubated for 3–4 days at 37°C in a humidified incubator and were slowly moved at least three times a day. After the completion of incubation period, the seven-day old eggs were observed under flash light to identify and encircle the embryo head. Thereafter, a tiny hole was drilled at the narrow end of the eggs and 0.5–1 ml of albumin was aspirated using eighteen-gauge hypodermic needle so that yolk sacs drop away from the shell membrane. The shell around the embryo air sac was detached via forceps and the shell membrane at the base of air sac was peel away. On 8th day, a Whatman No.1 filter paper loaded with 72.4 µg/ml of PPE sample was carefully placed on the surface of CAM and were incubated. After 3 days, the CAM was cut out from eggs and the numbers of vessels were observed. Vessels radially converging in the direction of the center were counted under a microscope. At least twenty eggs were used for each sample dose. The % of increase and inhibition were calculated.

DNA fragmentation assay

To confirm the mechanism of cell death mediated by PPE extract the DNA fragmentation assay was performed, which is characteristic for apoptosis. All cells were treated with PPE extracts at their concentrations such as 150 and 300 µg/ml, respectively for 48h and DNA was then isolated and analyzed by 2% agarose gel electrophoresis

RESULTS AND DISCUSSION

Antioxidant activity

Punica granatum peel ethanol extracts were studied by various free radical scavenging assays, including DPPH, FRAP and reducing power assays. The strong FRAP radical scavenging activity (32.5 µM) was noted in the 300µg/ml concentration of sample. The strong DPPH value (28.6%) was also recorded. The more reducing power assay (24.4 µM AAE/g) was observed in the 300 µg/ml concentration and followed by 200µg/ml (15.9 µM AAE/g). Among this, FRAP scavenging activity was showed the potential activity against the various concentration of PPE extract. The value was compared with standard ascorbic acid (Fig 1).

Cytotoxicity activity

Ethanol extract of *Punica granatum* peel was tested for the *in vitro* anticancer activity against HGT-1 cell line using the MTT assay. The cell line was cultured with PPE extract at concentrations in the range of 10µg/ml to 500µg/ml for 24 h and cell viability was determined. The minimum cell viability (27.29%) was observed in the 500µg/ml concentration of the PPE extract (Fig 2). The maximum cell inhibition was showed the increasing concentrations of PPE extract. In a dose depend manner, this extract inhibits the growth of HGT-1 cell. The IC_{50} value of ethanol extract of *Punica granatum* peel was 67.28µg/ml.

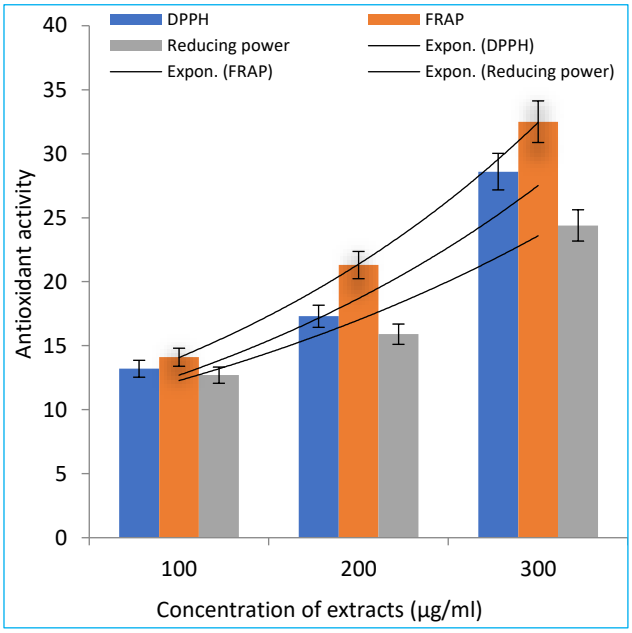


Fig 1 Antioxidant activities various concentration of PPE extract

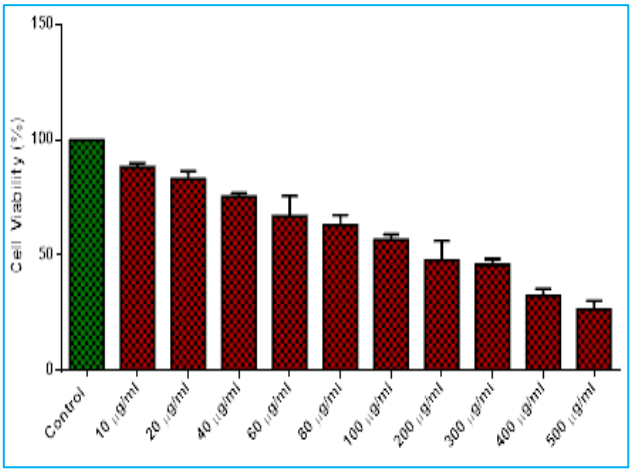


Fig 2 Anticancer activities of various concentration of PPE extract against HGT-1 cell line

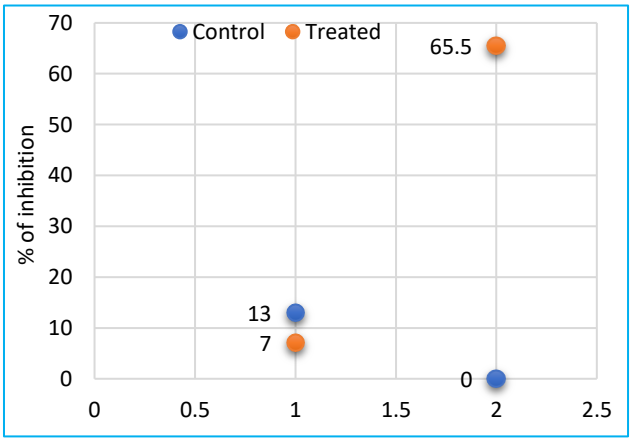
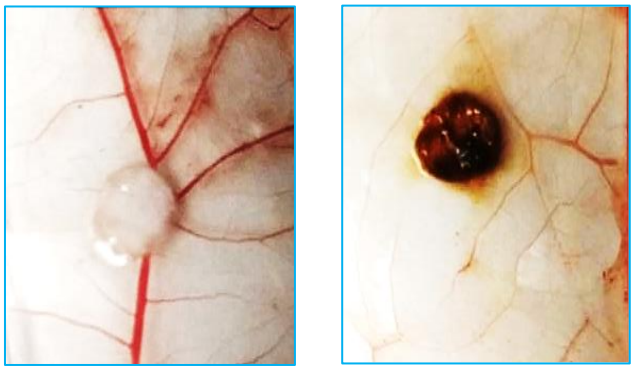


Fig 3 Anti-angiogenic activity of PPE on CAM assay

Anti -angiogenic activity

In vivo antiangiogenic effect PPE extract was also tested using CAM assay as an *in vivo* model at a dose of 72.4 µg/ml (Fig 3). The inhibitory activity for the *Punica granatum* peel ethanolic extracts with 65.50 ± 3.5% inhibitions was observed. The number of blood vessels (7) was reduced in treated group compared with control (13) (Fig 4).

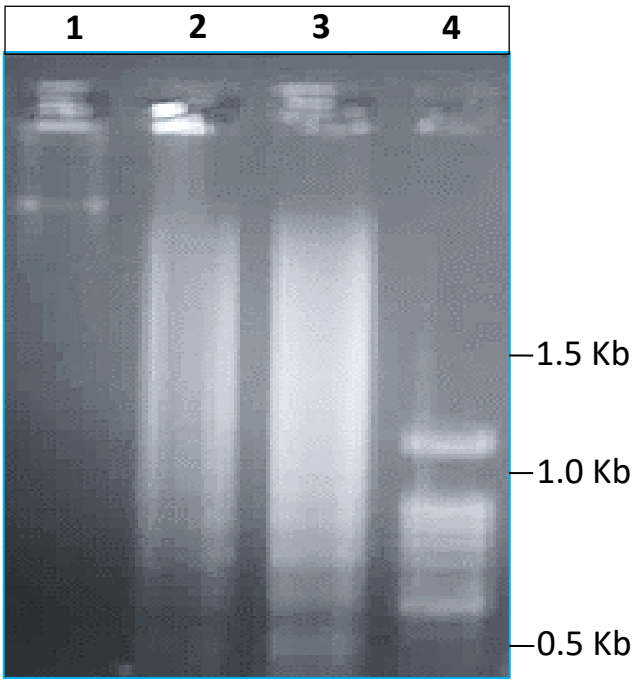


a) Control b) Treated with 72.4 µg/ml of PPE extract

Fig 4 Effect of PPE sample on angiogenesis in chick embryo CAM assay

DNA fragmentation assay

The DNA fragmentation assay was performed for the confirming of cell death. The apoptotic cells undergo DNA fragmentation which show ladder life pattern in agarose gel. The HGT-1 cell line was treating with two different doses of PPE extract while the control plates were left untreated for 24 hrs. Ethanol extract at both doses were induced apoptosis in the HGT-1 cells line as evidenced by the ladder like pattern of the DNA isolates from the treated plates compared to control. The banding pattern of treated sample was shown the DNA ladder. DNA laddering was observed when HGT-1 cell was incubated with 150 and 300 µg/ml of PPE extract for 20 h (Lane 1, 2, 3 and 4 stands for control, 150 µg/ml, 300 µg/ml and 100bp DNA marker respectively). Overall, these results suggest that the *Punica granatum* peel ethanolic extract effects fragmentation of DNA and thus induces significant apoptotic activity (Fig 5).



Lane 1- control HGT-1 cells, Lane 2- 150 µg/ml of PPE extract on HGT-1 cells, Lane 3- 300 µg/ml of PPE extract on HGT-1 cells, Lane 4 - DNA marker (100 bp)

Fig 5 DNA Fragmentation of HGT-1 cell line treated with different concentration of PPE extracts

Antioxidants protect cellular components from oxidative damage, which is likely to decrease risk of mutations and carcinogenesis and also protect immune cells, allowing them to maintain immune surveillance and response. The FRAP method is based on the capacity of polyphenols to reduce ferric iron Fe3+ to ferrous iron Fe2+. The reducing power is one of the ant oxidative mechanisms. Furthermore, the reducing capacity of a constituent can serve as an important indicator of its antioxidative potential [29]. The reducing activity may be due to polyphenols, such as flavonoids and anthocyanins. Some authors have reported that there is a direct correlation between antioxidizing activities and the ability of plant constituents to reduce ferric iron ($Fe3^{+}$) to ferrous iron ($Fe2^{+}$) [30]. In the present study, the PPE extract exhibited antioxidant properties as tested in DPPH and FRAP scavenging assays.

Cytotoxic agents can be of chemical substances, immune cells, proteins etc. On exposure to a cytotoxic agent the cells may lose its membrane integrity, stop dividing or undergo apoptosis [31]. There are several cytotoxic assay methods suitable for screening anticancer potency of substances. Here with used MTT method to measure the cytotoxicity of extract in terms of metabolic inactivity and necrotic death respectively. Generally secondary metabolites of medicinal plant strongly inhibit growth of cancer cell line. In the effective search of anticancer agents, the conception of fruits vegetables and medicinal plants exhibited reduced cancer risk, which was also scientifically proved [32]. Several studies have demonstrated that extract from plants have anticancer potential under in vitro as well as in vivo conditions [33]. Most of the studies related to evaluation of anticancer activity of plants showed that polar extracts are more potent in retarding cancer cell growth that that of non-polar extracts [34]. In the present study methanol extracts showed good inhibition capabilities of HGT-1 cell growth.

CAM assays have been widely used to study antiangiogenesis, tumor cell invasion and metastasis. In the earlier study stated that the *in vivo* CAM assay also illustrated the high impact of *S. triloba* against the newly formed vessel in the chicken embryonic membrane [35]. Many plants have been studied for their anti-angiogenic activity e.g., *Teucrium stocksianum* [36] *Argassum wightii* [37], and *Pleuro-tuseous* [38]. Angiogenesis is an energetic propagation and differentiation procedure, which have need of endothelial propagation, passage and tube development [39]. Tumors with physically powerful angiogenic action are associated with a lesser patient survival speed [40]. *G.lucidium* is a potent ant-angiogenic compound, targeting a number of key proteins in angiogenisis. To the best of our knowledge, these results represent the first line of evidence for a novel biological function for *G. lucidium* as an angiogenic inhibitor [41].

The DNA laddering technique is used to visualize the endonuclease cleavage products of apoptosis [42]. This assay involves extraction of DNA from a lysed cell homogenate followed by agarose gel electrophoresis. This result in a characteristic “DNA ladder” with each band in the ladder separated in size by approximately 180 base pairs. This methodology is easy to perform, has a sensitivity of 1 × 106 cells (i.e., level of detection is as few as 1,000,000 cells), and is useful for tissues and cell cultures with high numbers of apoptotic cells per tissue mass or volume, respectively. Since DNA fragmentation occurs in the later phase of apoptosis, the absence of a DNA ladder does not eliminate the potential that cells are undergoing early apoptosis. In this study also reported that the DNA fragmentation assay was used to determine whether the action of PPE extract was associated with apoptosis. Overall, these results suggest that the PPE extract was showed better effect on fragmentation of DNA and thus induces significant apoptotic activity.

CONCLUSION

Plant extracts can be used in combination with traditional chemotherapy or used as alternative sources for adjuvant cancer therapy, as some of these extracts have no adverse effects and activate the cells of the immune system. In our study the finding that *Punica granatum* peel extracts exhibited strong inhibitory activities against the HGT-1 cell line. The present studies concluded that the *Punica granatum* peel extract also exhibited antioxidant properties as tested in DPPH and FRAP scavenging assays. Our results also indicated that PPE is a potent ant-angiogenic compound, targeting a number of key proteins in angiogenisis. To the best of our knowledge, these results represent the first line of evidence for a novel biological function for *Punica granatum* peel as an angiogenic inhibitor. The induction of apoptosis by PPE extract was determined the DNA intercalating properties. This extract performed DNA double strand break and fragmented DNA. The present study concluded the *Punica granatum* peel extract showed a vital role of antioxidant and anticancer activities. Future work is needed to determine effects of the extracts investigated in this study on normal primary cells and on animals *in vivo*.

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Conflicts of interest

The authors declare no conflict of interest.

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