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Growth Inhibitory and Pro-Apoptotic Effects of Ornamental Pomegranate Extracts in Du145 Human Prostate Cancer Cells

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ABSTRACT

Aims: Prostate cancer is the most common form of cancer in the male. Epidemiological studies have associated increased cancer incidence with reduced consumption of fruit and vegetables. This study was aimed to investigate the influence of dwarf pomegranate extracts (peel, juice, and seeds oil) on the proliferation and apoptosis of human prostate androgen-independent cell line DU145.

Methods: The cell viability was determined by the MTT assay. Morphological changes are detected by light microscopy. The ELISA assay is used to evaluate the nuclear DNA fragmentation and western Blot to detect the expression of apoptosis-associated proteins including poly-ADP-ribose polymerase (PARP) and cyclooxygenase-2 (COX-2).

Results: The three tested extracts exhibited a dose-response cytotoxic effect and antiproliferative action on DU145 cell line and induce morphological changes. The dose of each extract required to inhibit cell proliferation by 50% (IC_{50}) was 0.12, 0.36, and 0.42 mg/mL, respectively, for seeds oil, juice, and peel. The three extracts could also induce prostate cancer cell apoptosis by an increase of DNA fragmentation, PARP cleavage, and inhibition of the COX-2 expression. The strongest pro-apoptotic effect was shown after peel treatment.

Conclusion: Dwarf pomegranate extracts exhibited potent growth inhibitory activities in human prostate cancer cells (DU145), which appear to be mediated by a pro-apoptotic mechanism.

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Introduction

Cancer is the leading cause of death in the twenty-first century. According to the American Cancer Society, prostate cancer (PC) is the most common form of cancer in male, accounting for 1 in 5 new cancer diagnoses in men and ranking the second leading cause of cancer-related mortality (1). Compelling risk factors involved in the development and progression of prostate cancer include both genetic and environmental factors (2). In this context, increasing interest has centered on nutritional factors that offer protection against PC or increase its incidence (3). In fact, epidemiological studies have associated increased cancer incidence with reduced consumption of fruit, vegetables, and fish oil and relatively high intake of red meat. Moreover, dietary chemoprevention of PC is increasingly considered to be an important way to reduce this health problem (4–6). The evidence for a protective effect of a greater vegetable and fruit consumption is consistent for various types of cancer like tomato for PC (7) and berry fruits for colon cancer (8).

For the pomegranate, several scientific studies have confirmed its biological activities and medicinal effects (9). Different pomegranate parts (arils, peels, leaves, and flowers) and products (fresh and fermented juices, enriched extracts, and seeds oil) contain strong antioxidant activity, with potential health interests. In fact, most of the plant's therapeutic properties are due to the presence of natural bioactive compounds like polyphenols, tannins, and anthocyanins (10) which are reported to trigger apoptosis in a lot of cancer cells with different histologic origins (11, 12). So, tremendous attention was paid to pomegranate extracts as a natural alternative for the treatment of a wide range of cancers (13). The most of phytochemistry and pharmacological studies were interested in edible pomegranate varieties. But, no research was carried out on ornamental types. Hence, our study was aimed at exploring the *in vitro* effects of peel; juice extracts,

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and seeds oil of non-edible pomegranate variety (*Punica granatum*. L. *var* .*Nana*) in human PC androgen-independent cell line DU145. We evaluated the antiproliferative and pro-apoptotic effects of the three extracts.

Materiel and Methods

Plant Material

Pomegranate fruits were harvested from dwarf trees from Moknine region, Tunisia. The variety authenticity was confirmed by taxonomist Pr Messaoud Mars from the Department of Horticulture, Higher Agronomic Institute (University of Sousse, Tunisia).

Fruits were washed and hand-peeled. Arils were squeezed using a commercial blender (Moulinex, France). The extracted juice was centrifuged at 15,000 rpm for 15 min. Then the supernatant was recuperated and lyophilized. Peel and grains were sundried and powdered.

Extraction

Lyophilized juice and peel powder were extracted with methanol 50 g/250 mL in the dark for 48 h. Each extract was filtered and evaporated to dryness and stored at –20 °C for further determination. Oil was extracted by the methods of soxhlet as described previously (14). From each extract, solutions of final concentrations ranging from 0.01 to 1 mg/mL were prepared by serial dilutions using dimethyl sulfoxide (DMSO).

Cell Lines, Cell Culture and Treatment and Light Microscopy

The DU145 cell line was purchased from the American Type Culture Collection (LGC Standards, Middlesex, UK). Cells were seeded at 2×10^6 cells in 60 cm^2 tissue culture dishes and grown in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin and 100 μg/mL streptomycin (all from Gibco-BRL, Cergy-Pontoise, France). Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were grown for 24 h in culture medium prior to exposure or not to different pomegranate extracts. A stock solution of each extract was prepared in DMSO and diluted in culture medium to give the appropriate final concentration. The same amount of vehicle (percentage of DMSO did not exceed 0.2%) was added to control cells. Cell viability was determined by the trypan blue dye exclusion method. For light microscopy, cultured cells were examined under phase-contrast microscopy (magnification, ×200), 24 h and 48 h after treatment and pictures were taken with an image acquisition system (Nikon, Champigny sur Marne, France).

Cell Proliferation Assay

The effects of pomegranate extracts on the viability of DU145 cells were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells (8×10^3/well) were plated in 100 µl of medium/well in 96 well plates. After overnight incubation, pomegranate extracts were added at various concentrations (0.01–1 µg/mL). Controls were performed with the same final DMSO concentrations in media as in samples. After treatment with pomegranate extracts or DMSO for 24 h and 48 h, 10 µl of 5 mg/mL MTT was added to each well and incubated for another 3 h. The supernatant was removed and 100 µl DMSO was added per well. Samples were then shaken for 5 min. The absorbance at 550 nm was measured with a microplate reader (Bio-Rad, Marnes-la-Coquette, France), using wells without cells as blanks. All experiments were performed in triplicate. The effect of peel, juice, and seeds oil on the proliferation of cancer cells was expressed as relative cell viability. The IC_{50} value was the concentration of extract which inhibited cell growth by 50% compared to controls.

Apoptosis Quantification: DNA Fragmentation

DU145 cells were seeded at 2×10^6 cells in 60-cm^2 tissue culture dishes and then treated or not with pomegranate extracts for 48 h. Apoptosis was quantified on pooled cells (floating and adherent) using 'cell death' enzyme-linked immunosorbent assay (ELISA) (Cell Death Detection ELISAPLUS, Roche Diagnostics, Basel, Switzerland). Cytosol extracts were obtained according to the manufacturer's protocol and apoptosis was measured as previously described (15).

Protein Extraction and Western Blot Analysis

After 48 h treatment, the total cell pool (attached and floating cells) was centrifuged at 200 g for 5 min at 4 °C and homogenized in RIPA (50 mM HEPES, pH 7.5, 150 mM NaCl, sodium deoxycholate1%, NP-40 1%, SDS 0.1%, 20 mg/mL of aprotinin) containing protease inhibitors (CompleteTM Mini, Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. Proteins (10–100 µg) were separated by electrophoresis on SDS-PAGE gels and

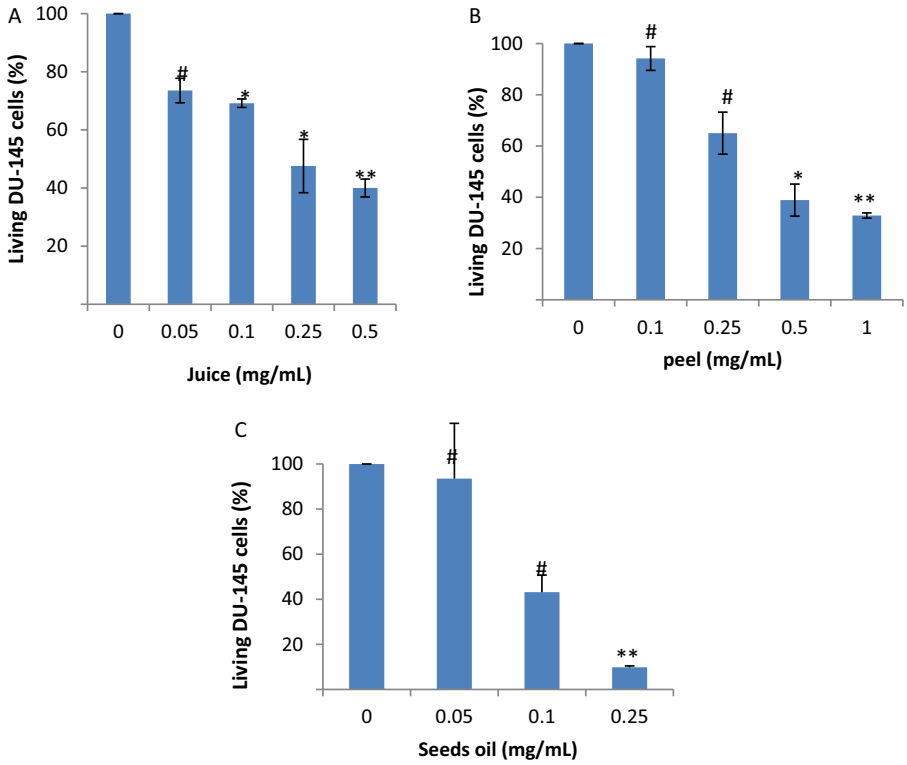

Figure 1. A. Effect of juice extract, B. peel extract, and C. seeds oil treatment on DU145 cell viability.

transferred to polyvinylidene fluoride (PVDF) membrane (Amersham, GE Healthcare Life Sciences, Paris, France) and probed with human primary antibodies at 4 °C overnight. Human antibody against PARP and COX-2 (16) according to the manufacturer's instructions was purchased from Santa Cruz Biotechnology (Clinisciences, Nanterre, France) and Cayman Chemical (Bertin Pharma, Montigny-le-Bretonneux). After incubation with secondary antibodies (Invitrogen, Thermo Fisher Scientific, Illkirch, France and Cell Signaling Technology), blots were developed using the "ImmobilonWestern" (Millipore, Saint-Quentin-en-Yvelines, France) and G:BOX system (Syngene, Ozyme). Membranes were then reblotted with anti-β-actin (Sigma, Saint-Quentin Fallavier, France) used as a loading control.

Statistical Analysis

All results are presented as means ± SD of three replications (three individual extracts per each fruit) and statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) program, release 16.0 for Windows (SPPS, Chicago, IL). IC_{50} was determined by one-way ANOVA using Tukey's test. The statistical significance was defined to be at a level of $p < 0.05$.

Results and Discussion

Effect on Cell Proliferation and Morphology

To evaluate the anti-proliferative effect of various extracts, human PC cell line (DU145) was treated with designated concentrations of seeds oil (0.05, 0.1, and 0.25 mg/mL), juice extract (0.05, 0.1, 0.25, and 0.5 mg/mL), and peel extract (0.1, 0.25, 0.5, and 1 mg/mL) for 24 h and 48 h, respectively. This evaluation is done via the formazan reduction test (MTT).

The best anti-proliferative effect was obtained after 48 h of treatment for all extracts. As revealed in Fig. 1, various pomegranate extracts incubated for 48 h inhibited DU145 cell proliferation in a concentration-dependent manner. The best anti-proliferative effect was observed after seeds oil treatment (Fig. 1c). At 0.25 mg/mL, it exhibited more than 80% of cell growth inhibition. However, relatively high amounts of peel (Fig. 1b) and juice (Fig. 1a) extracts were needed for to inhibit cell growth by 70% (1 mg/mL and 0.5 mg/mL of peel and juice extracts, respectively). In accordance with our results, Deng et al. (2017) found that low concentrations of pomegranate peel extract (PoPx); prepared from Tunisian soft-seed pomegranate variety, have no effect on DU145 cell viability. However, the higher concentrations and the longer time treatment showed good inhibition of cell growth (12). According to Li et al. (2016), PoPx has a

Table 1. Relative antiproliferative activity of pomegranate extracts in cancer cell line DU145.

Extract	IC_{50} (mg/mL)
Oil	0.12 ± 0.023
Juice	0.36 ± 0.021
Peel	0.42 ± 0.04

IC_{50}: concentration that inhibit growth of cell by 50% vs control cells. Data are presented as the mean ± SD. Three separated experiments were carried out for each extract.

relatively high selectivity index and its IC_{50} value on normal cells was more than 400 mg/mL, which indicated that PoPx might have high safety profiles (17). Although juice and seeds oil seem to be more effective, low concentrations are sufficient to inhibit cell proliferation. In fact, according to Table 1, the highest anti-proliferative activity against DU145 cells was detected after 48 h in the presence of seeds oil with an IC_{50} value of 0.12 mg/mL, compared to control (untreated cells), followed by juice extract with an IC_{50} value of 0.36 mg/mL. The least important anti-proliferative activity was recorded in the presence of peel extract with an IC_{50} value of 0.42 mg/mL.

The anti-proliferative effect of pomegranate's juice, peel, and seeds oil against PC was also demonstrated in other varieties of pomegranate. In fact, a study on the anticancer effect of the edible variety "Wonderful" has shown that these three extracts are potent inhibitors of the proliferation of human cancer cell lines LNCaP, PC3, and DU145 (2).

Further, the exposure of DU145 cells to different extracts of the pomegranate fruit for 48 h at 0.1 mg/mL, 0.5 mg/mL, and 0.5 mg/mL for seeds oil, peel, and aril juice, respectively altered cellular morphology (Fig. 2). In fact, direct observation with phase-contrast microscopy demonstrated that cells treated with extracts showed numerous morphological differences compared to control cells. Indeed, cell shrinkage, cytoplasm condensation and formation of cytoplasmic filaments appeared after extracts treatment. Furthermore, the pictures of treated cells confirmed that the anti-proliferative effect was more important with seeds oil and peel treatment.

Several previous studies have shown that this fruit has great anti-proliferative potential against PC by reducing the invasion of cancer cells and inhibiting their growth both *in vitro* and *in vivo*. The anti-proliferative effect recorded in each extract can be attributed to specific compounds such as polyphenols and fatty acids. In fact, we have demonstrated in our previous study that different parts of dwarf pomegranate tree are rich in phenolic compounds such as anthocyanins, flavonoids, and carotenoids (18). In addition, another Tunisian research about the same *Nana* pomegranate variety has identified 21 phytochemical compounds by HPLC coupled to both DAD and ESI/TOF-MS detections. Among the detected compounds, anthocyanins, ellagitannins, ellagic acid derivatives, and flavanols were further characterized by MS-MS (19). Indeed, Albrecht et al. (2004) tested the *in vitro* effect of polyphenols extracted from the pomegranate fruit against three PC cell lines LNCaP, DU145, and PC3 and they discovered that these macromolecules are able to reduce significantly proliferation and cellular invasion (2). In addition, Malik and Mukhtar (2006) reported that ellagic acid extracted from pomegranate is endowed with anti-proliferative and pro-apoptotic properties causing both, inhibition of cell growth and apoptosis in a dose-dependent manner in PC3 cells (20).

Pomegranate seeds oil is rich in compounds endowed with anticancer activity, including unsaturated fatty acids, sterols (β-sitosterol, stigmasterol, and campesterol) and tocopherols (14, 18). Some of them have chemopreventive properties against cancer. For example, punicic acid C18:3 (c9, t11, c13), a conjugated fatty acid and the main constituent of edible pomegranate varieties (70–80%), exhibited potent growth inhibitory activities in androgen-dependent LNCaP cells, which appear to be mediated by both anti-androgenic and pro-apoptotic mechanisms [3]. We have demonstrated previously in a comparative study between edible pomegranate and dwarf pomegranate that seeds oil of Nana variety contained lesser amounts of Conjugated linolenic acids (Clna) (2.81%), especially punicic acid (18).

Effect of Pomegranate Extracts on DNA Fragmentation

To test whether the decrease in cell viability by the extracts was due to the induction of apoptosis, we evaluated the nuclear DNA fragmentation of DU145 cells after 48 h of exposure to the three extracts of the pomegranate fruit in a complete culture medium. It is well known that apoptosis is characterized by chromatin condensation and DNA fragmentation, and is mediated by the cysteine protease family called caspases (21). In Fig. 3, we note that the treatment of DU145 cells by the various pomegranate extracts caused an increase in DNA fragmentation. Indeed, the results of the apoptotic ratio that actually reflects the amount of DNA degraded, shows a significant difference between treated and untreated cells. The largest amount of fragmented DNA is recorded in the cells treated with the peel extract with a value of 5-fold,

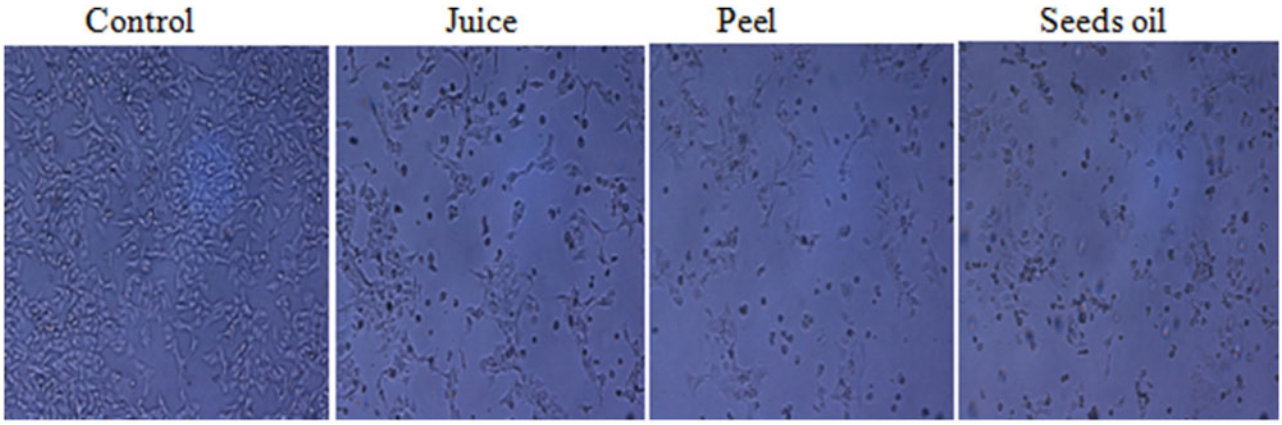

Figure 2. Effect of extracts treatment on DU145 cell morphologic changes and proliferation.

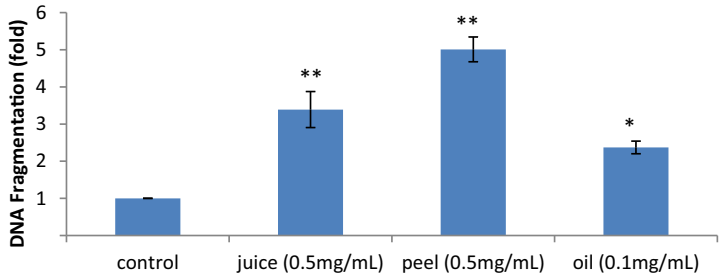

Figure 3. Apoptosis was quantified on floating and adherent cells using 'cell death' ELISA on the basis of DNA fragmentation.

followed by those treated with the juice extract (3.4-fold) and finally with the seed oil (2.3-fold). It should be noted that the concentration of the oil used in this case (0.1 mg/mL) is 5 times lower than that of juice and peel (0.5 mg/mL).

So it turned out then that the peel extract seems to have the most important pro-apoptotic effect. These data are similar to that found in edible pomegranate varieties. In fact, a recent study conducted by Deng et al. (2017) showed that pomegranate peel extract induced cell apoptosis and inhibit migration and invasion (12). Moreover, the study conducted by Song et al. (2016) suggested that pomegranate peel polyphenols extractions induced human hepatoma cell apoptosis by a mitochondrial pathway in a dose-dependent manner (22).

Effect of the Various Extracts on PARP Cleavage and COX-2 Expression

To gain more insight into the precise mechanism of pomegranate extracts to induce apoptosis, respectively, two markers of apoptosis and survival pathway, cleavage of PARP and COX-2 expression were evaluated by Western Blot after 48 h of exposure to the three extracts. Results were illustrated in Fig. 4.

When compared to control, the tested extracts induced a PARP cleavage. Indeed, a disappearance of the bands corresponding to the native PARP (112 kDa) while others corresponding to the cleaved PARP (85 kDa) appeared. Intense bands corresponding to cleaved PARP were remarked in cells treated with the peel. This result correlates with the important amount of fragmented DNA recorded in the cells treated with the peel.

On the contrary, COX-2 is an enzyme that allows the formation of prostaglandins from arachidonic acid. Numerous studies have shown that COX-2 expression prevents apoptosis in cancer cells, especially in the colon (23) and prostate cancer (24, 25). Recently, we showed that *Berberis libanotica* extract reduced significantly the expression of COX-2 by a dose-dependent manner in HEL and K562 (COX-2+) erythroleukemia cells (26). Here, we showed that the expression of COX-2 is reduced in treated DU145 cells compared to control cells. All extracts strongly inhibit the COX-2 expression without any difference, confirming probably the equal anti-inflammatory property, promoting the apoptotic effect, of all pomegranate extracts tested on DU145 human PC cell line. In regard to our results, it is clear that the simultaneous cleavage of PARP and inhibition of COX-2 expression can significantly contribute to the

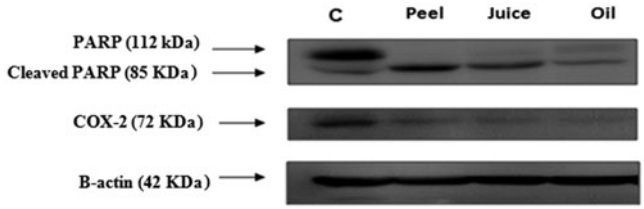

Figure 4. Effect of pomegranate extracts on PARP and COX-2 expression.

anticancer effects of the three pomegranate extracts in human PC cells.

Notably, these observations are in accordance with recent studies conducted with pomegranate bioactive compounds or extracts. For example, ellagic acid (EA), the major compound of pomegranate juice or peel, induced apoptosis in LNCaP cells, by increasing Bax/Bcl-2 ratio and caspase 3activation. Moreover, cell-cycle-related proteins expression, p21 (WAF), p27 (Kip), cdk2, and cyclin E were increased while cyclin D1 and cdk1 were decreased by EA treatment (27). The apoptotic activity of pomegranate seeds oil can be attributed to the presence of several bioactive molecules that have shown a potent inhibitory effect on cell growth, especially in the case of PC. In fact, punicic acid has been shown to be an effective inducer of apoptosis in LNCaP cancer cells. It allows the activation of caspase-9, which activated caspase-3, degrading PARP, in turn, thus allowing the degradation of DNA (3). In addition, γ-tocopherol, a molecule abundant in pomegranate oil, induces apoptosis in cancer cells, but not in normal cells as it inhibits COX-2 activity in PC cancer cells (28).

Conclusion

We could clearly presume that juice, peel, and seeds oil of pomegranate Nana variety have an anti-proliferative effect and anti-cancer activities against PC cell lines, especially DU-145. We have demonstrated that all extracts of this non-edible variety displayed strong cytostatic effects on DU-145 cells. The three pomegranate extracts tested could also induce PC cell apoptosis by induction of PARP cleavage, DNA fragmentation, and inhibition of COX-2 expression. Considering the reported effects and the nutraceutical properties of the different extracts (peel, juice and seeds oil), we could suggest that this ornamental variety of pomegranate might be developed as a novel approach to reduce PC.

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Authors' Contributions

Amri Z. designed the study, participated in analysis, interpretation, and writeup, drafted the manuscript and critically revised the manuscript. Kharroubi W participated in analysis, interpretation, and write-up. Fidanzi-Dugas C. and David Y. L. participated in study design and analysis, provided materials and reagents. Hammami M. drafted and critically revised the manuscript. Bertrand L. provided materials and reagents, drafted, and critically revised the manuscript. All authors read and approved the final manuscript.

Disclosure Statement

The authors declare that there are no conflicts of interest regarding the publication of this article.

Data Availability Statement

The dataset used and/or generated during the current study are availablefrom the corresponding author upon request.

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