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Research Article

Combination of Pycnogenol and Melatonin Reduce PC-3 and HT29 Cell Migration: Comparison to the Actions of Cisplatin

Ali Taghizadehghalehjoughi[1]*, Sidika Genc[2], Yesim Yeni[2], David R Wallace[3], Ahmet Hacimuftuoglu[2] and Zeynep Cakir[4]

[1]Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology, Ataturk University, Erzurum, Turkey

[2]Faculty of Medicine, Department of Medical Pharmacology, Ataturk University, Erzurum, Turkey

[3]Oklahoma State University Center for Health Sciences, School of Biomedical Sciences, Tulsa, Oklahoma, United States

[4]Faculty of Medicine, Department of Emergency Medicine, Ataturk University, Erzurum, Turkey

***Corresponding Author:** Ali Taghizadehghalehjoughi, Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology, Ataturk University, Erzurum, Turkey.

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Abstract

Prostate and colorectal cancer are among the most common malignant tumors. The aim of the current study is to evaluate the effects of melatonin (MLT) and pycnogenol (PYG) combination on PC-3 and HT-29 cancer lines and to address the question of where or not MLT increases PYG antitumor effect in the PC-3 and HT-29 cancer lines. The combination of 100 µg/ml PYG + 40 µg/ml MLT reduced cell viability in both HT-29 and PC-3 cells (66 and 65%, respectively). Results from the TAC, TOS and LDH assays were correlated to our MTT results. In addition, the cellular migration test showed that the wound line was widened in the combination groups starting at 72 hours compared to the other groups. Cisplatin has routinely used as an anticancer agent, but because of side effects, usage is limitation. The combination of 40 µg/ml MLT with PYG increased the extent of antitumor effects compared to Cisplatin and reduced the cell viability effectively than Cisplatin. The combination of MLT + PYG shows promise to be a new anticancer agent for the treatment of HT-29 and PC-3 patients or adjuvant for the reduction of the side effects of chemotherapeutics.

Keywords: HT-29; Melatonin; PC-3; Pycnogenol

Introduction

Antioxidants are substances that can neutralize free radicals and prevent cell damage. Epidemiological studies show that the long-term consumption of antioxidants can protect us against a range of diseases, such as cancers, cardiovascular diseases, diabetes, osteoporosis, and neurodegenerative diseases (e.g. Alzheimer's disease) [1-4].

Pycnogenol (PYG) is an extract from the bark of the French maritime pine Pinus pinaster Ait. PYG is known for its antioxidant, anti-inflammatory and antitumor promotional properties in a variety of experimental models. PYG includes different components such as flavonoids, taxifolin, catechin and epicatechin, polyphenolic monomers, and cinnamic acids. Because of these components, PYG has antioxidant activity. It has been reported that PYG has exhibited

protective effects in many chronic diseases including cancer, obesity, dyslipidemia, inflammatory, immune system disorders, and diabetes [5-8]. Multiple *in vitro* studies have also substantiated the *in vivo* studies and have reported that PYG has anticancer activity in a variety of malignant cell lines, including leukemia, ovarian, and breast, and fibrosarcoma cancer cells [9-12].

Melatonin (N-acetyl-5-methoxy-tryptamine) is the main product of the mammalian pineal gland [13]. Generally, Melatonin (MLT) has effects on the regulation of a variety of physiological and pathological processes, including antioxidation, anti-aging, anti-inflammation, anti-angiogenesis, stimulation of cell differentiation, and activation of the immune system [14-16]. In recent years, many experimental studies have shown that melatonin has an antitumor effect *in vitro* cancer types such as prostate cancer [17], colorectal cancer [18] breast cancer [19], lung carcinoma [20], esophageal cancer [21] and pancreatic cancer [22].

Cisplatin has been especially interesting since it has shown anticancer activity in a variety of tumors, including those of the prostate and colon. Although patients with such cancers have recently received better prognosis for survival with a reduction in mortality, significant challenges remain the development of a cure. Also, because of drug resistance and significant side effects, combination therapy of cisplatin with other cancer drugs have been applied as novel therapeutic strategies for many human cancers.

Prostate cancer (PC) is the second most common malignant tumor that begins in the outer part of the prostate. The third most common malignant tumor is colorectal cancer (CRC). Colorectal cancer has had a low incidence until a few decades ago. Since then it has become dominant cancer and currently accounts for about 10% of cancer-related deaths in western countries and more than 0.5 million deaths worldwide [23]. Many risk factors, including smoking, alcohol consumption, dietary factors, lifestyle, ethnicity, and genetic changes, have been associated with PC and CRC [24]. For PC and CRC patients, early diagnosis and chemotherapy, surgical resection, radiotherapy, or targeted therapies are applied clinically [25]. However, the agents currently used for cancer treatment are usually cytotoxic and have serious side effects that can reduce the quality of life of cancer patients [26]. Many epidemiological studies have shown that dietary habits, including antioxidant consumption, significantly decreased PC and CRC development [27-29].

Although there are many separate studies in the literature regarding the actions of MLT and PYG, there is limited research on the combined treatment, and no data on the potential antitumor effects of MLT and PYG in combination [30,31]. For the first time, in this study, we evaluated the antitumor effects of MLT and PYG combination therapy in PC-3 and HT29 prostate and colorectal tumor cells.

Aim of the Study

For this aim, we designed the current study to consist of 13 separate treatment groups. The antitumor study was done using MTT, TAC, LDH, cell migration assay (wound healing test) and morphological observation for 72 hours.

Materials and Methods

Plant material, chemicals and reagents

PYG was obtained from Horphag Research Ltd., Guernsey (UK-USA). MLT was obtained from Swanson Health Products (Fargo, ND, USA). All chemicals, Dulbecco Modified Eagles Medium (DMEM), Fetal calf serum (FCS), phosphate buffer solution (PBS), antibiotic antimitotic solution (Penicillin/Streptomycin/Amphotericin B) (100×), L-glutamine and trypsin–EDTA were obtained from Sigma Aldrich (St. Louis, MO, USA) CSP was obtained from Koçak Farma (Tekirdag, Turkey).

Cell cultures

Prostate (PC-3) (ATCC® CRL-1435) and colon (HT-29) (ATCC® HTB-38) cancer cells were obtained from the department of medical pharmacology department of Ataturk University (Erzurum, Turkey). Briefly, the cells were centrifuged 1200 rpm for 5 minutes at the room temperature were suspended in fresh medium and then aliquoted into 24-well plates at a density of 10^5 cells/ml (DMEM, with FBS 10%, B27 2% and antibiotic (Penicillin, Streptomycin and Amphotericin B; 1%) and allowed to grow in a humidified incubator with 5% CO_2 at 37°C [32].

Plant administration

After cells reached 85% confluency, the plant extracts were added to corresponding treatment wells. PYG (10, 20, 40, 60, 80 and 100 µg/ml) alone or in combination PYG (10-40-80-100 µg/ml) and MLT (40 µg/ml) was added to designated treatment wells, and the plates were returned to the incubator for 72 hours.

Cell viability

3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) assay: At the end of the experiment (after 72 hours of treatment), 10 µL of MTT solution is added to each well plate (1 mM final concentration). Then the plates were incubated for 4 hours at 37°C in a CO_2 incubator. After 4 hours, 100 µL of DMSO solution was added to each well to dissolve the formazan crystals. The density of

the formazan crystals was read at a wavelength of 570 nm by the Multiskan™ GO Microplate Spectrophotometer reader [32].

Total oxidant status (TOS) and total antioxidant capacity (TAC) assay: TOS and TAC evaluation were done by measuring spectrophotometrically (Multiskan™ GO Microplate Spectrophotometer reader) the density of the color relevant to the number of oxidants in the sample. We used the commercially available TOS (Total Oxidant Status) kit (manufactured by Rel Assay Diagnostics® Company, Gaziantep, Turkey).

To determine the TOS, 500 µl Reactive 1 (Buffer solution, H_2SO_4) solution was added to the wells in which the 75 µl cell medium sample was present and after reading the initial absorbance value at 530 nm, 25 µl Reactive 2 (Substrate Solution, H_2SO_4, Ferrus ion, O-dianisidine) solution was added in the same well. The second absorbance was read at 530 nm at the end of the waiting period of 10 minutes at room temperature [32].

To determine the TAC, 500 µl Reactive 1 (Buffer solution, Acetate Buffer) solution was added in the wells containing 30 µl of sample, and the first absorbance was read at 660 nm. Then, 75 µl Reactive 2 (Prochromogen Solution, ABTS) was added to the same wells and allowed to wait at room temperature for 10 minutes. At the end of the waiting period, the second absorbance value was read at 660 nm [33].

Lactate dehydrogenase (LDH) assay: LDH assay test was performed using a commercially available test kit from Cayman Chemical Co. Ltd, (Ann Arbor, MI, USA). Briefly, the cell culture medium was centrifuged at 400g for 5 minutes at the room temperature 100 µl of the supernatant was added to 100 µl of the reaction solution (LDH Assay Buffer, LDH Substrate Mix) and incubated with gentle shaking on an orbital shaker for 30 minutes at room temperature. Finally, the absorbance was read at 490 nm wavelength.

Migration test (Wound healing test)

Cells were seeded in 24-well plates and incubated (37°C, 95% air/5% CO_2) for 3 days. Cultures at ~80% confluence were scraped with a micropipette tip (100 - 200 µl), rinsed with phosphate-buffered saline (PBS), and incubated with fresh media. At 24, 48 and 72h intervals, photomicrographs were obtained using a Leica microscope [34].

Statistically analysis

The statistical analysis was done using one-way analysis of variance (ANOVA)to assess treatment effects followed by the Tukey's HSD for posthoc comparisons when appropriate using the SPSS 22.0 statistical software. $P < 0.05$ was considered to be the statistical threshold for each analysis.

Results

Cell viability

MTT analysis

The MTT assay results were shown in figure 1a and 1b. The survival percentages of the other groups were also calculated by comparing absorbance values of the different treatment groups to control values, with control values set as 100% viability. The smallest effect among treatment groups compared to control was seen in the CSP (2 µg/ml) group (19% reduction compared to controls, $P > 0.05$). The survival rate using PYG alone (10- 20- 40- 60- 80 and 100 µg/ml) decreased in a concentration-dependent manner from 78% to 71% in HT-29 cells (Figure 1a), and from 80% to 72% in PC-3 cells (Figure 1b). The addition of MLT to PYG was more effective than PYG alone in both cell lines. The optimum combination of 100 µg/ml PYG + MLT (40 µg/ml) significantly reduced cell viability in both HT-29 and PC-3 cells (66 and 65%, respectively). Also, all of the MLT + PYG combination groups showed a statistical difference compared to the control group ($P < 0.05$).

TOS and TAC analysis

We determined TOS content which was standardized to H_2O_2 Equiv/mmol L^{-1} (Figure 2a and 2b). The oxidant level of the control group was 2.28 and 2.92 H_2O_2 Equiv/mmol L^{-1} in HT-29 and PC-3 cells, respectively. The lowest oxidant ratio among the treatments was seen in the CSP group. In the presence of PYG only, oxidant status increased in both cell lines ($P < 0.05$). The effect of increased oxidant status was also observed in the groups receiving a combi-

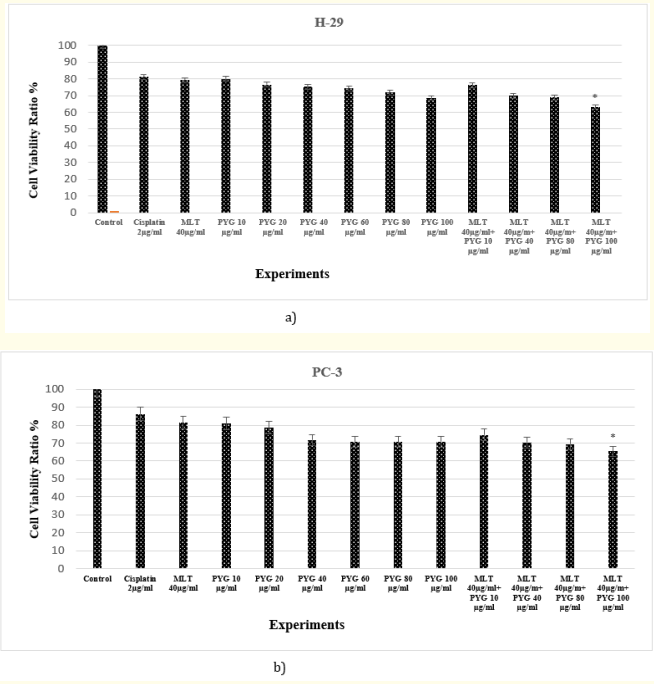


Figure 1: *In vitro* viability ratio of melatonin, pycnogenole or combination on HT-29 (a) and PC-3 (b) cells (n = 6/group). *Significant differences at P < 0.05 compared to control group; **Significant differences at P < 0.001 compared to control group.

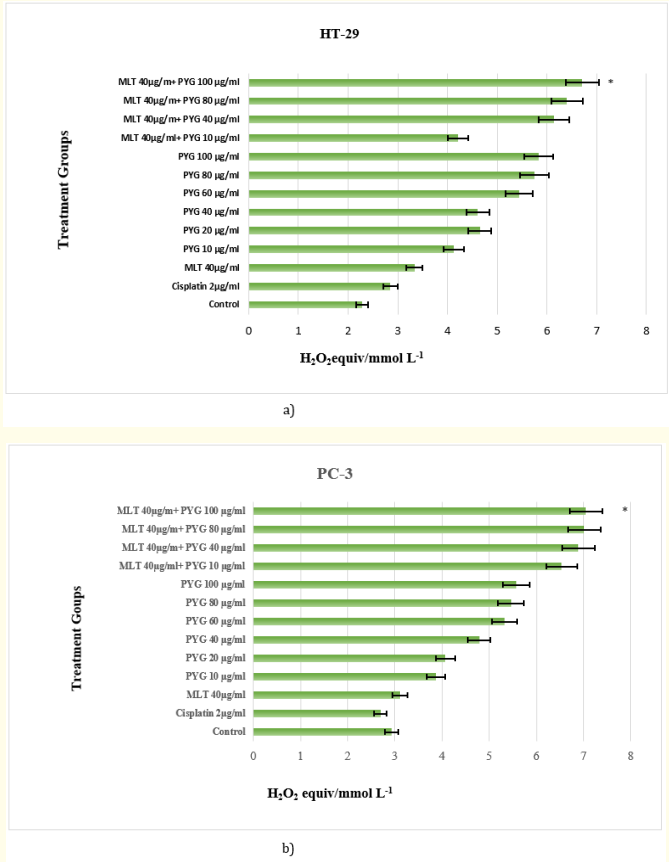


Figure 2: *In vitro* TAC capacity of melatonin, pycnogenole and combination of them on HT-29 (a) and PC-3 (b) cells (n = 6/group). *Significant differences at P < 0.05 compared to control group; **Significant differences at P < 0.001 compared to control group.

nation of PYG with MLT in both cell lines. The combination of MLT 40 µg/ml + PYG 100 µg/ml maximally increased total oxidant ratio to 6.71 and 7.05 H_2O_2 Equiv/mmol L^{-1} in the HT-29 (Figure 2a) and PC-3 (Figure 2b) (P < 0.05).

TAC content was standardized to Trolox Equiv/mmol L^{-1} (Figure 3a and 3b). In the control group, TAC levels were 6.72 and 6.22 Trolox Equiv/mmol L^{-1} in HT-29 and PC-3 cells, respectively. TAC levels in the CSP group (2 µg/ml) was 6.63-6.09 and TAC levels in the MLT alone (40 µg/ml) group ranged from 6.29-5.80 in the HT-29 and PC-3 cells, respectively (Figure 3a and 3b). TAC was inversely related to increasing concentrations in the PYG group, resulting in decreasing TAC. PYG exposure at concentrations of 10 to 100 µg/ml (2.04 in the HT-29 and 0.72 in the PC-3 Trolox Equiv/mmol L^{-1}) resulted in significant reductions in TAC content compared to control values. In the MLT (40 µg/ml) + PYG (100 µg/ml) group was found to be significantly reduced compared to the control group (P < 0.05). There no statistically significant differences observed between the other groups of lower concentrations of PYG compared to control values (P > 0.05).

LDH analysis

Damage to the cell membranes is reflected as elevated LDH levels in the cell medium after the cells were exposed to MLT (40 µg/ml), PYG (10- 20- 40- 60- 80 and 100 µg/ml) or MLT + PYG (10-40-

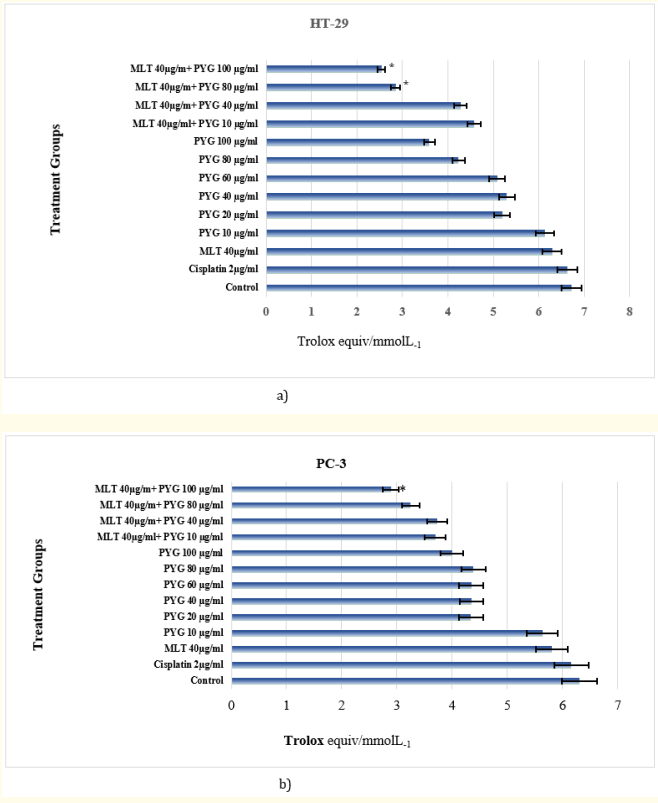


Figure 3: *In vitro* TOS status of melatonin, pycnogenole or combination on HT-29 (a) and PC-3 (b) cells (n = 6/group). *Significant differences at P < 0.05 compared to control group; **Significant differences at P < 0.001 compared to control group.

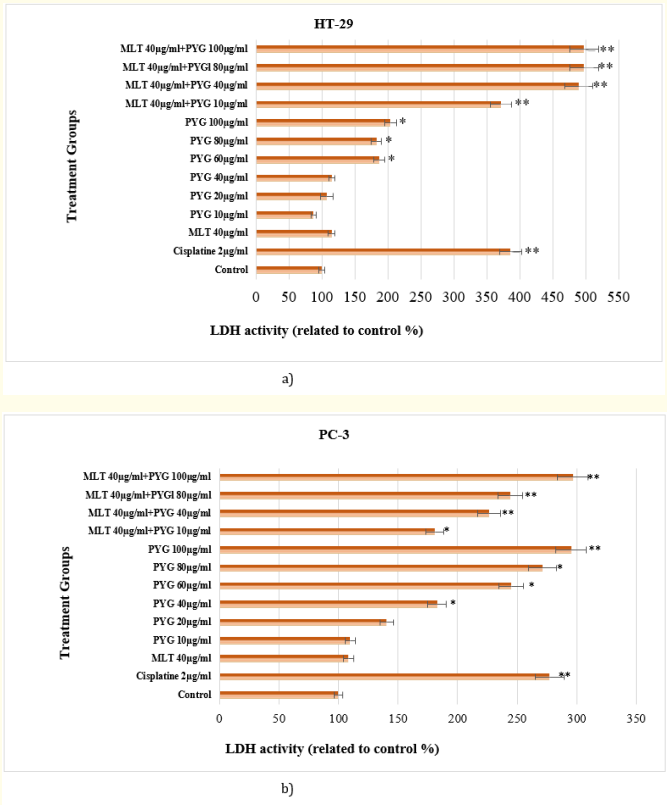
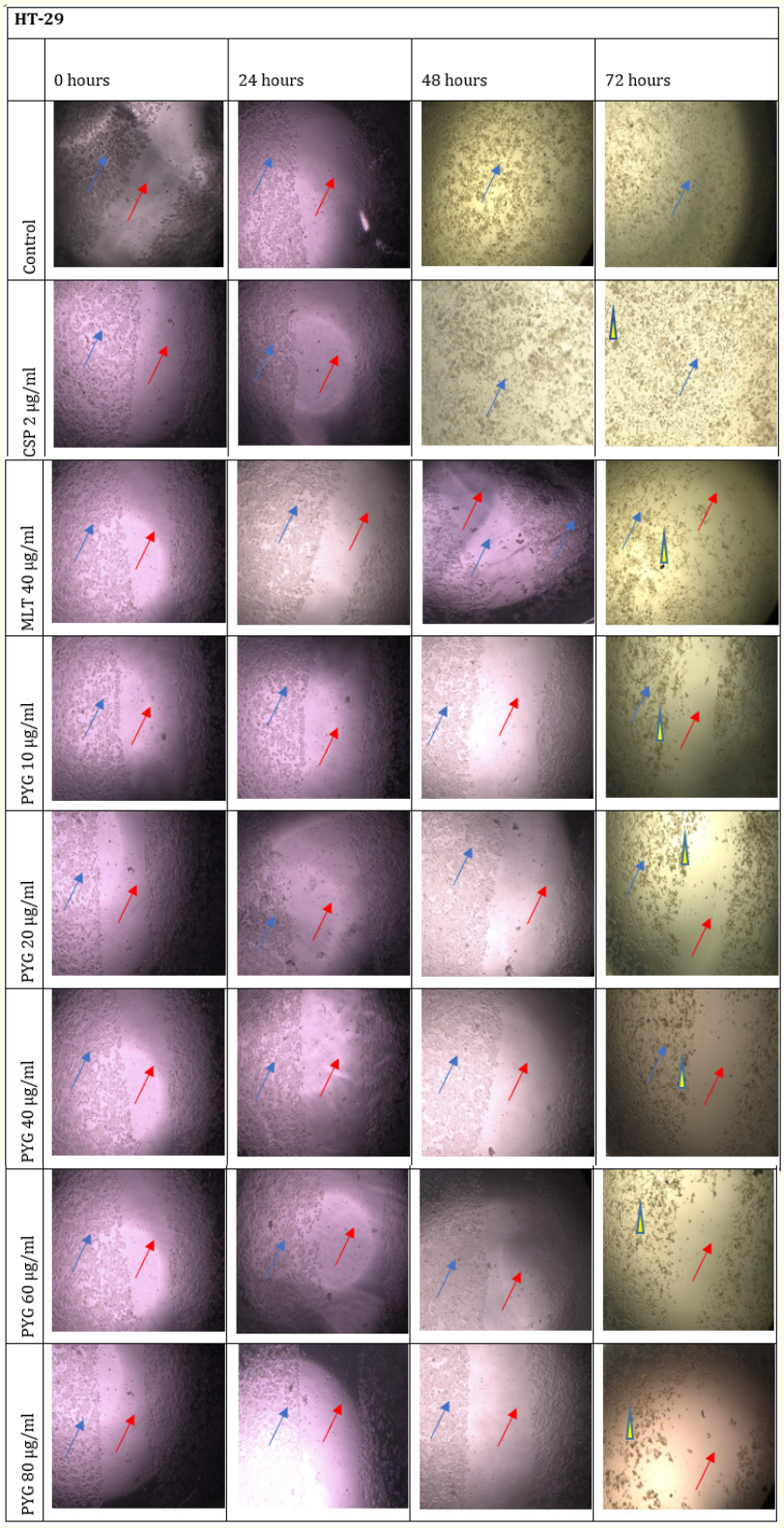


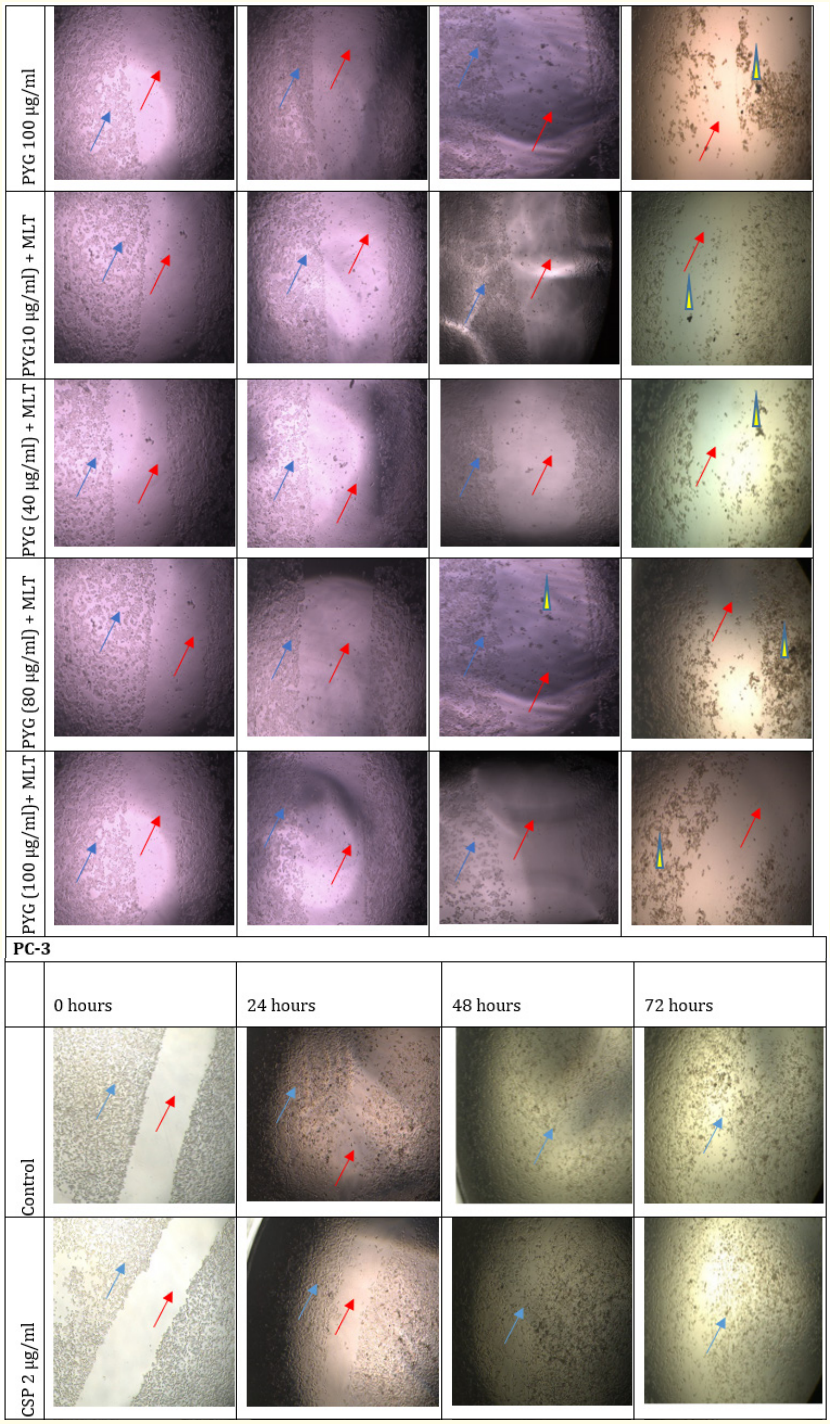
Figure 4: *In vitro* lactate dehydrogenase (LDH) level of melatonin, pycnogenole or combination on HT-29 (a) and PC-3 (b) cells (n = 6/group). values read spectrophotometrically at 490 nm in cell culture. *Significant differences at P < 0.05 compared to control group; **Significant differences at P < 0.001 compared to control group.

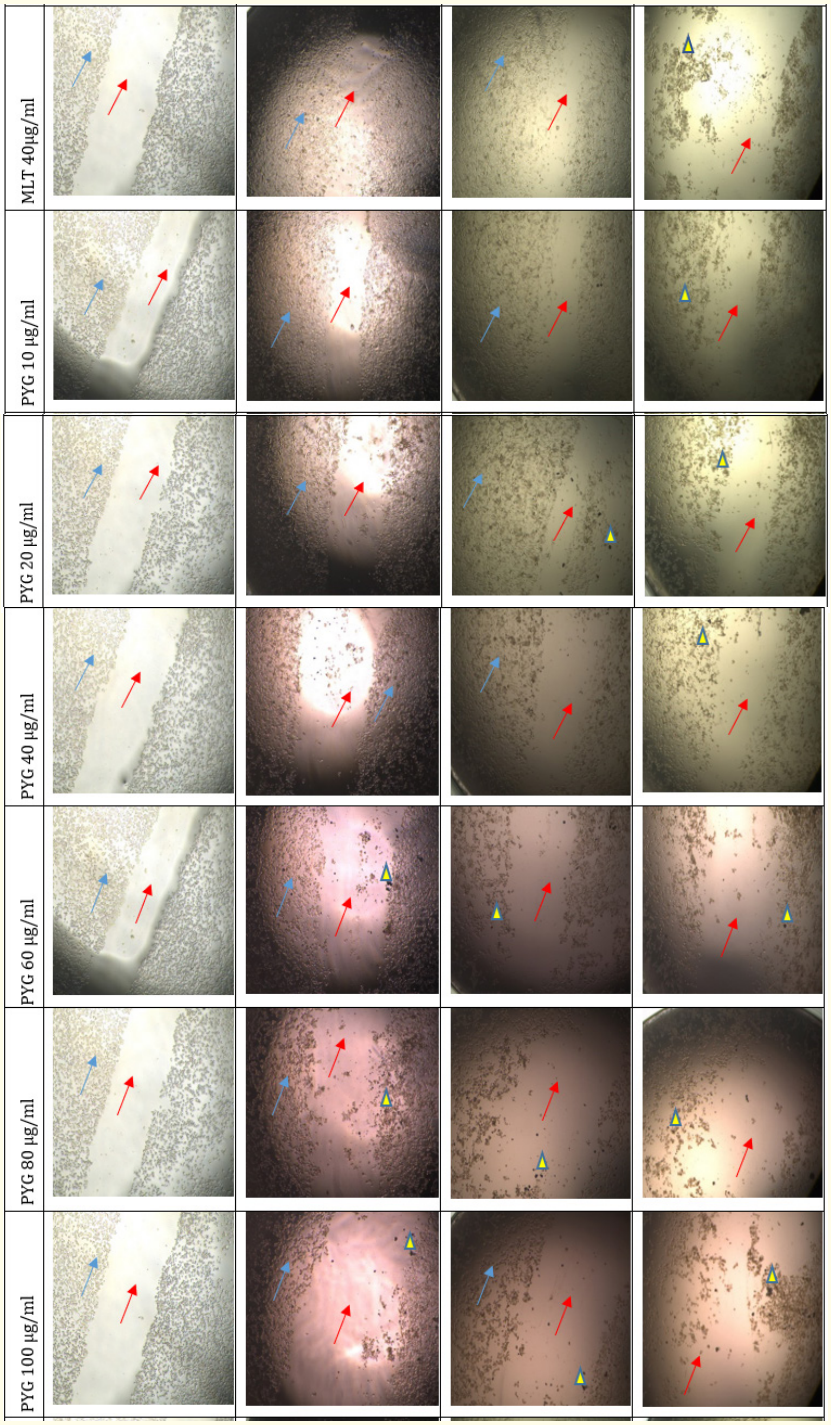
80 and 100 µg/ml) combinations for 72h. The LDH activity of the control group was defined as 100%, and the other groups were rated accordingly. Our results show that the MLT (40 µg/ml) + PYG (100 µg/ml) combination was most toxic as indicated by the greatest amount of LDH activity in the media from the PC-3 cell line in comparison to other treatments. When examining the HT-29 cell line, the combination of MLT (40 µg/ml) + PYG (40-80 and 100 µg/ml) had the highest LDH activity ratio compared to other treatments. Figure 4a and 4b shows that the MLT+ PYG combinations induced cytotoxicity in both PC-3 and HT-29 cell lines in a time- and dose-dependent manner. All of the MLT + PYG combination groups showed statistical differences when compared to the control group (P < 0.001).

Migration test (Wound healing test)

Wounds opened in HT-29, and PC-3 cell cultures using a 100 - 200 µl pipette tip are shown in figure 5a and 5b. In the first 24 hours, it was observed that the cells started to close the opened regions in the control group, while no change was detected in the other groups. After 48h, the control group progressed further and closed all wound areas. Similar to the control group, the CSP group showed a large reduction in wound area, and the migrating cells bridged the wound site. Cell migration from the wound line to the middle of the wound was observed in the MLT 40 µg/ml and PYG 10


HT-29
0 hours
24 hours
48 hours
72 hours
Control
CSP 2 µg/ml
MLT 40 µg/ml
PYG 10 µg/ml
PYG 20 µg/ml
PYG 40 µg/ml
PYG 60 µg/ml
PYG 80 µg/ml


PYG 100 µg/ml
PYG10 µg/ml) + MLT
PYG (40 µg/ml) + MLT
PYG (80 µg/ml) + MLT
PYG (100 µg/ml)+ MLT
PC-3
0 hours
24 hours
48 hours
72 hours
Control
CSP 2 µg/ml


MLT 40µg/ml
PYG 10 µg/ml
PYG 20 µg/ml
PYG 40 µg/ml
PYG 60 µg/ml
PYG 80 µg/ml
PYG 100 µg/ml

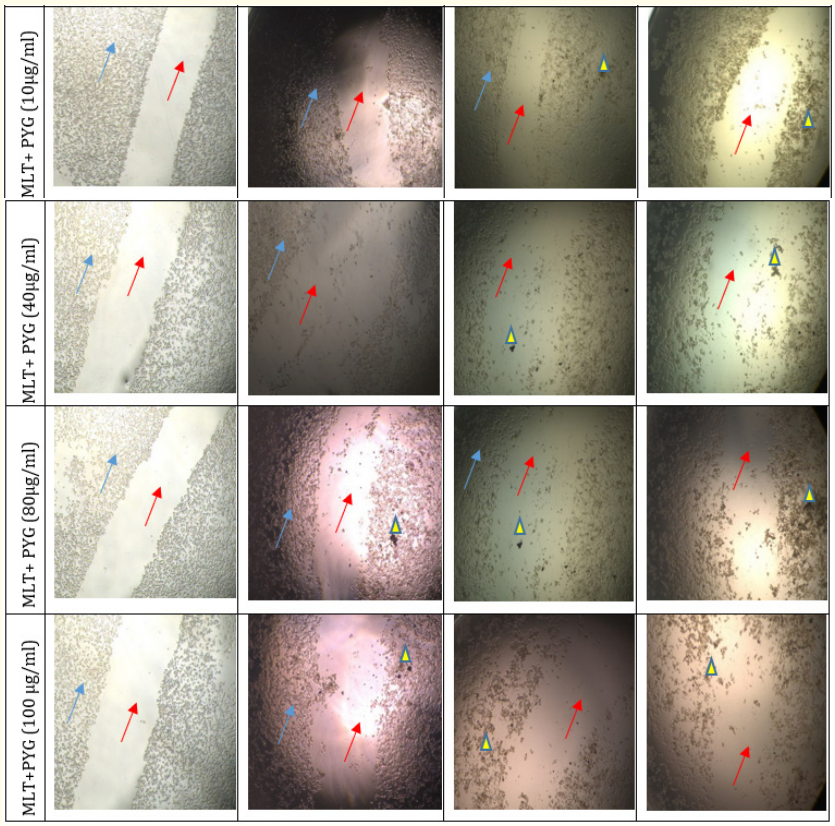


Figure 5a and 5b: Microscopic wound model (migration test) view of each group after 24, 48, and 72-hour (20×). Pyramid: empty aera, Blue arrow: Cell line and Red arrow: Wound line.

µg/ml group. However, the wound line was widened with increasing concentrations of PYG from 20 to 100 µg/ml. Evidence of cellular death was observed and after 72h, we observed that the wound lines were completely closed in the control and CSP groups. However, in all treatment groups, including MLT 40 µg/ml and PYG, 10 µg/ml high rates of continued cellular death was observed. In all combination groups, the widening of the wound and the lack of cell migration due to cell death was greater compared to other single treatment groups and controls.

Discussion

In the present study, the antitumor effects of the antioxidants, MLT and PYG, as well as the combination of MLT + PYG therapy was studied in the HT-29 and PC-3cell line using LDH, MTT, TOS, TAC and Migration Test analysis, and morphologic determination. Chemotherapy is one of the most commonly used therapeutic methods for cancer treatment; however, most anticancer drugs are cytotoxic to normal cells [35]. The cytotoxicity in normal cells tends to limit the usefulness of some chemotherapeutic agents, and is a source of critical adverse effects. Various studies have shown that antioxidants have antitumor activity [3,36,37] and have given new hope for increasing the patient's quality of life or lifespan. Therefore, we investigated the antitumor effects of the plant-derived compound, PYG, MLT and the combination of MLT+ PYG.

In a clinical study with 64 chemotherapy patients, PYG reduced the adverse effects associated with standard oncological treatment.

In this study, 25 patients receiving PYG showed a decreased frequency of essentially all investigated side-effects as compared to 21 patients receiving placebo. The most significant improvements occurred with the side effects of pain and ulceration in the mouth and throat, as well as less drying of the mouth and eyes. It has been observed that nausea, vomiting, diarrhea, edema, and weakness decrease [36].

Huang., *et al*. have studied the antitumor effects of PYG in three leukemia cell lines (HL-60, K562 and U937) [10]. They found that PYG inhibited cell proliferation both dose- and time-dependently. The authors concluded that PYG-induced apoptosis is mainly mediated by the activation of caspase-3 [38]. In our study, we showed that combination MLT + PYG inhibits cell proliferation in both HT-29 and PC-3 cell lines by reducing antioxidant capacity and increasing oxidant status. Increasing in LDH level and oxidant status (free radicals) can damage DNA and cell material therefore decrease viability and proliferation potential [39]. Our data showed MLT+PYG is more effective than CSP and individual exposures to MLT or PYG due to the increased cellular LDH release and oxidant status. In addition, the level of LDH and oxidant status are different in both cell lines. This difference originates from the unique ability of antioxidant capacity. Our data demonstrated that the antioxidant capacity of PC-3 is higher than HT29. We observed that the viability of PC-3 was higher than in the HT29 cell lines following antioxidant exposure.

Huynh and Teel studied selective induction of apoptosis in human mammary cancer cells (MCF-7) by PYG [9]. In this study, they compared apoptotic responses in human breast cancer cells (MCF-7) and normal human mammary cells (MCF-10). They report that PYG exposure did not significantly alter the number of apoptotic cells in MCF-10 cells (DAPI staining) compared to the MCF-7 tumor. However, PYG exerted its anticancer effects through the suppression of cytochrome P450 enzyme activity and the induction of apoptosis [39]. Melatonin exerted its antiproliferative effects via the upregulation of melatonin MT1 and MT2 receptors and p53-dependent activation. These mechanisms are safer than the cisplatin-induced DNA damage due to the widespread effects of the DNA damage and the resulting development of drug resistance following long-term administration. The combination of MLT+PYG works through different mechanisms compared to cisplatin, that can result in cellular death without the cisplatin-associated side effects. In addition, our data has demonstrated that the combination of MLT+PYG may elicit synergistic effects compared to MLT and PYG alone in both HT-29 and PC-3 cell lines [9].

A study by Harati., *et al*. investigated the apoptotic effect of PYG on human fibrosarcoma cells (HT1080) [12]. For this purpose, vital, apoptotic and necrotic cells were quantified using flow cytometry, and gene expression analyzed by RNA microarray. Their results show remarkable expression changes induced by PYG in a variety of genes, which are involved in various apoptotic pathways of cancer cells [Janus kinase 1 (*JAK1*), *DUSP1*, *RHOA*, laminin γ1 (*LAMC1*), fibronectin 1 (*FN1*), catenin α1 (*CTNNA1*), *ITGB1*]. Furthermore, morphological changes (cellular collapse or the development of deformities) were observed in this study [12]. The data from Harati., *et al*. is correlates with the data we have presented in the curent study. We did not see metastasis into the open wound area (Figure 5a and 5b); the cells were spheroid (without poda), and the number of deformed cells are elevated compared to control positive and negative groups. Administration of MLT and PYG alone had therapeutic effects, but the combination of different MLT concentrations significantly increased the PYG antitumor effect. Our theory is that the effect augmentation is due to, and dependent on, stress induction following stimulation of the MLT-1 and MLT-2 receptor. After MLT receptor stimulation, PYG (20 and 40 μg/ml) increased movement into tumor cells resulting in an increased aggregation of intracellular PYG molecules. Our studies showed that the MLT (40 μg/ml) + PYG (100 μg/ml) combination induced cell damage and morphological changes in both cell lines more effectively than CSP or other treatment groups. As shown in figure 5, increasing PYG concentrations resulted in the wound line remaining open, with no metastasis, unlike the control and cisplatin groups where cell migration closed the wound gap. Also, the combination of MLT+PYG elicited effects that were visible 24 and 48 hours after exposure started, whereas other treatment groups did not show appreciable effects until 72h after initiating exposure. The rapid effects that we have observed in the present study would suggest that MLT+PYG combination results in an additive or synergistic effect than the treatments alone, and that the enhanced effect may lead to improved cancer therapeutics.

Yang., *et al*. [10] investigated the role of apoptosis-inducing factor (AIF) and caspase-independent apoptosis as mediators of the PYG antitumor effect in MC-3 cells. The authors concluded that PYG exposure resulted in a concentration-dependent release of AIFinto the cytosol as well as the nucleus from the failing mitochondria during apoptosis. The release of AIF from damaged mitochondria following PYG exposure was confirmed by immunohistochemical (IHC) labeling. They also suggested that PYG might be a potent caspase-independent apoptotic inducer in MC-3 cells. According to previous reports, PYG showed anti-MC-3 activity by inducing apoptosis via activation of AIF release. Similarly, our study demonstrated a concentration-dependent reduction in viability following PYG exposure. Figure 1 shows the response to increasing concentrations of PYG and the reduction in MTT staining. Lower staining reflects a reduction in the activity of NAD (P)H-dependent oxidoreductases, and a subsequent reduction in the insoluble formazan production. Reductions in the oxidoreductases, a mitochondria-associated enzyme, is interpreted as a loss of viable cells. Therefore, in the present study we report a reduction in staining and a loss of viability suggesting mitochondrial dysfunction leading to impaired mitochondrial respiration and ATP generation. The affects observed were only at the higher concentrations examined [40].

Conclusion

Side effects and chemotherapeutic resistance to cisplatin are significant and pose major health concerns for the patient. Specially, irreversible DNA damage, neuropathic pain and vital organ damage has led us to find better approaches to cancer treatment that will minimize or by-pass these significant side effects. In working towards this end of better therapeutics, we utilized the plant flavonoid\ (PYG) and a hormone, melatonin (MLT) that has reported anticancer properties. In our study, we compared the effects of MLT and PYG alone and in combination, and measured cellular viability, oxidative stress response and cell migration as markers for anticancer-related effects in HT-29 and PC-3 cells. The combination of MLT + PYG shows promise s a new anticancer agent for the treatment of colorectal or prostate cancer or adjuvant for the reduction of the side effects of chemotherapeutics. PYG and MLT together augmented the anticancer effects of each compound alone, not only by inducing oxidative stress, direct or indirect apoptosis (AIF) but also by direct effect on M1 and M2 receptors. By targeting both the MLT receptors and mitochondrial function, this would be an avenue for the development of new therapies for the treatment of prostate and colorectal cancer. Also, work is being done to investigate whether the combination of these agents along with CSP will permit the lowering of the CSP dose and the resulting reduction in CSP-mediated side effects.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, or the preparation and scientific review of the contents and approval of the final version of the article.

Animal and Human Rights Statement

No animal or human studies were carried out by the authors for this article.

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