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Synergistic Anticancer Effect of Melatonin and Ascorbyl Palmitate Nanoformulation: A Promising Combination for Cancer Therapy

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Abstract: Combining anticancer drugs enhances treatment outcomes and achieves superior therapeutic effects in cancer treatment. The present study aimed to assess the anticancer effect of melatonin (MEL) and ascorbyl palmitate-loaded pluronic nanoparticles (APnp) combination on Ehrlich ascites carcinoma (EAC)-bearing mice. Tumor volume, viability, and evaluation of histopathological, ultrastructural, and immunohistochemical studies were investigated. Furthermore, cell cycle and western blotting analysis were also carried out to explore the anticancer action of the MEL and APnp combination. The present study shows for the first time that a combination of MEL and APnp can synergistically reduce tumor growth accompanied by increased antioxidant profiles and decreased levels of oxidative stress. It also induced apoptosis and DNA damage. Besides, mediated cell cycle arrest. Moreover, the IL-6/STAT3 pathway was inactivated to a greater extent after our combination treatment. The results of the immunohistochemical examination demonstrated the antiproliferative effect of MEL and APnp *via* decreased expression of Ki-67. Our combination of MEL and APnp was able to inhibit cancer cell invasion and metastasis by decreasing the protein expression of MMP-9. Finally, we conclude that the combination of MEL and APnp is considered a novel therapeutical strategy for cancer treatment as it shows superiority over MEL alone.

Keywords: melatonin; ascorbyl palmitate; anticancer; nanoparticles; STAT3; MMP9; cancer.

1. Introduction

Cancer is a deadly disease categorized by the uncontrolled cell proliferation and spread of abnormal cells through invasion or metastasis [1]. Cancer therapy involves surgery, radiotherapy, and chemotherapy, the major conventional cancer treatments [2]. These treatments' dangerous side effects and restricted efficiency have forced researchers to discover novel effective anticancer drugs based on natural products and herbal extracts, either single or combined therapies [3-7]. Many strategies are being used to control this dangerous disease, including using nanoformulations of natural products or their semi-synthetic derivatives. Natural compounds are always regarded as safe treatment options [8].

Melatonin (MEL), also known as (N-acetyl-5-methoxytryptamine), is a naturally occurring hormone produced by various tissues in the human body. It is primarily produced by the pineal gland but also by other organs such as bone marrow, retina, gastrointestinal system, and lymphocytes [9,10]. As an antioxidant agent, melatonin scavenges free radicals and inhibits oxidative stress *in-vitro* and *in-vivo* [11,12]. Melatonin is a lipophilic compound that has a broad spectrum of biological anticancer effects, including anti-angiogenic [13], anti-migration, anti-invasion [14,15], pro-apoptotic [16], and anti-proliferation activities [17]. According to previous reports, MEL exerts anticancer roles in several types of cancers, including cervical, gastric, lung, colorectal, and breast cancer [18-22].

Ascorbyl palmitate (AP) is one of the most important derivatives of ascorbic acid and has been used as a source of vitamin C. It is an amphipathic compound due to esterification with palmitic acid [23-25]. In addition, AP acts as an antioxidant molecule and exerts antitumor activity *via* its antiproliferative effect [26,27]. AP is found to be a more stable ascorbate derivative than free ascorbate, but its poor release capacity and water insolubility limit its bioavailability and therapeutic efficacy [28,29]. Therefore, it can be incorporated into pharmaceutical nanocarriers, such as pluronic, which has excellent amphiphilic and biocompatibility properties to enhance circulation time and accumulation of drugs in the tumor by the enhanced permeability and retention (EPR) effect [30,31]. Recently, we have shown that ascorbyl palmitate-loaded pluronic nanoparticles (APnp) could target and kill EAC tumor cells *via* several target pathways, and we indicated the superiority of nanoformulation of AP as a promising anticancer agent over native AP [26].

Here, we investigated the synergistic anticancer effect of a novel combination of melatonin and APnp as a promising potential therapy in cancer treatment against EAC-bearing mice.

2. Materials and Methods

2.1. Drugs and chemicals.

Melatonin (MEL, $C_{13}H_{16}N_2O_2$, and MW 232.28g/mol) and ascorbyl palmitate (AP, $C_{22}H_{38}O_7$, and MW 414.5 g/mol) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Pluronic F-108 (MW 14600 g/mol) was provided by Sigma-Aldrich (Germany). Acetone 99.9% and all other reagents and solvents were of analytical grade and used as received.

2.2. Preparation of Ascorbyl palmitate nanoparticles.

AP-loaded pluronic nanoparticles (APnp) were prepared *via* the nanoprecipitation method recently described by El-Far et al. [26]. Briefly, 50 mg of (AP) and 150 mg of (F-108) were dissolved in 5 and 15 mL acetone, respectively, followed by mixing the two solutions. Pluronic (F-108) was added to the drug (AP) solution in a 1:3 ratio (drug: polymer). The drug (AP)/F-108 acetone solution was added dropwise into 50 mL of deionized water. Afterward, the resulting nanoparticles were frozen at -80^{o}C and freeze-dried (FreeZone 6, Labconco) at -50°C and stored for further use.

2.3. Characterization of Ascorbyl palmitate nanoparticles.

AP-loaded pluronic nanoparticles (APnp) were fully characterized by Fourier transform infrared spectroscopy (FTIR), transmission electron microscopy (TEM), UV-vis spectroscopy,

and Zeta potential analysis, as reported by El-Far et al. [26]. APnp were spherical, with an average size of 220.0±9 nm, Zeta potential of -41.16±2.0 mV, PDI of 0.1, and a high encapsulation efficiency of 88.51 ± 1.1%.

2.4. Animals and experimental design.

Thirty adult female Swiss albino mice weighing 20–25 g were used in the present study. They were housed under standard conditions and were fed the standard nutrition and water ad libitum. The current study was approved by Mansoura University Animal Ethical Committee (Code no. Sci-Ch-ph-2022-168). The EAC cell line was inoculated by intraperitoneal (IP) passages of 1×10^6 cells per mouse. EAC cells were grown in the peritoneal cavity of mice [32]. The experimental mice were divided into 3 groups (Table 1), each containing 10 mice. All groups were injected with 1×10^6 EAC cells/mouse (0.2 mL of EAC cell/mouse, IP). This was taken as day 0. Ascetic fluid was collected from each mouse after 2 days of the last given dose.

Table 1. Distribution of groups in the study.

Groups	Treatment (6 separate doses)
Group I	Only EAC, without any treatment.
Group II	EAC+ MEL alone (25 mg/kg, IP, day/day).
Group III	EAC+ combination of MEL and APnp. On days 1, 5, and 9, mice received MEL(25 mg/kg, IP), and on days 3,7, and 11, mice received APnp (25 mg/kg, IP).

2.5. Tumor volume and viability percentage and synergy effect analysis.

Ascetic fluid was collected from the peritoneal cavity of the experimental mice. Then tumor volume was measured, and viability percentage was assessed by Trypan blue exclusion method [33]. After obtaining the present data on cell viability, the synergy score was calculated by the web application SynergyFinder 2.0 using four reference models with the "cell viability readout" [34].

2.6. Biochemical measurements of oxidative parameters.

All oxidative parameters were evaluated in the prepared EAC homogenates [35]. The levels of oxidative stress were estimated by measurement of catalase (CAT), superoxide dismutase (SOD), reduced glutathione (GSH), and lipid peroxidation (MDA) using assay kits from Biodiagnostic, Egypt. Furthermore, the amount of nitric oxide (NO) was assessed by Griess assay using the supernatant of ascetic fluid [36].

2.7. Histopathological studies.

Ascites fluids were collected from different groups, then centrifuged, and processed normally like other tissues (liver, kidney, and spleen); fixed in 10% buffered formalin pH=7.2, embedded in paraffin followed by sectioning, staining with H&E and finally examined using Olympus Bx 51 light microscope.

2.8. Transmission electron microscopic studies.

Collected tissues were immediately fixed using 4% formaldehyde: 1% glutaraldehyde fixative (4F:1G) and 1% osmium tetroxide prepared in phosphate buffer. After that, ascending ethanol series was used to dehydrate specimens to be embedded in the resin. Staining with lead citrate and uranyl acetate followed by ultrathin sectioning with an ultramicrotome to be examined by JEOL electron microscope (Japan) at 60 kv.

2.9. Comet assay.

The Comet assay is considered a sensitive, simple, rapid, and visual method for assessing DNA damage and the early stages of apoptosis [37]. The EAC tumor cells of mice from all groups were used for the damage evaluation of DNA by the method described by Rageh *et al.* [38]. Comets were examined using a fluorescence microscope. The DNA damage was analyzed, and the tail moment was obtained using Image J software *via* OpenComet v1.3.1 plugin [39].

2.10. Cell cycle analysis.

EAC cells were fixed with 70% ice-cold ethanol and washed with cold PBS. Finally, the cells were resuspended in PI based on the method of El-Far *et al.* [33]. EAC cells were analyzed using BD FACSCCalibur™ Flow Cytometer (Becton Dickson, Mountain View, CA).

2.11. Western blot.

The EAC cell lysate from different groups was prepared using RIPA lysis buffer containing a cocktail of protease inhibitors and phosphatase inhibitors for immunoblotting of STAT3 (1:1000, Cell Signaling Technology, Cat No. 9139T), p-STAT3(Tyr705) (1:2000, Cell Signaling Technology, Cat No. 9145T) and MMP-9 (1:2000, Cell Signaling Technology, Cat No. 3852S). Western blot analysis was carried out as previously reported [26].

2.12. Immunoenzymatic Assay.

IL-6 was evaluated through immunoenzymatic assay (ELISA) using a supernatant of ascites fluid washing [40].

2.13. Immunohistochemical studies.

Sections of EAC cells were processed standardly using blocking agents like absolute methyl alcohol of 0.5% H_2O_2 and 0.4% HCl for suppressing endogenous peroxidase action. Sections were then washed with (PBS) 3 times and then rinsed in the serum-blocking buffer. Afterward, sections were incubated at 4°C with primary antibodies, Ki-67 antibody (Thermo Fisher Scientific, Cat No. MA5-14520), and Bcl-2 antibody (Thermo Fisher Scientific, Cat No. MA5-11757). A secondary antibody was then applied to slides to be stained with recommended chromogen. Mayer's hematoxylin stain was used as a counterstain for investigated slides. The scoring protocol was applied according to El-Far *et al.* [26].

2.14. Statistical analysis.

All the values were expressed as mean ± SE. Statistical analysis was performed through SPSS software 26.0 using One-Way ANOVA with GraphPad Prism 5.0. $P< 0.05$ was

considered statistically significant, p < 0.01 was considered statistically highly significant, and p < 0.001 was considered statistically extremely significant.

3. Results

3.1. Antitumor activity.

Antitumor activity of MEL alone or in combination with APnp against EAC tumor-bearing mice was evaluated by parameters including tumor volume and percentage viability, as shown in Figure 1. A highly significant (P<0.01) reduction in both tumor volume and viability was observed in MEL-alone treated mice when compared to the EAC control group. The tumor volume and viability significantly decreased (P<0.001), and thus the number of nonviable cell counts was increased on treatment with MEL in combination with APnp. We demonstrated that MEL alone showed a decrease in tumor growth by 48%, while in the case of using MEL combined with APnp, it displayed inhibition of tumor growth by 62%. Furthermore, we reported that the combination of MEL and APnp synergistically reduced the growth of the tumor. The synergy score was 21.06 (> 10 indicates synergistic effect).

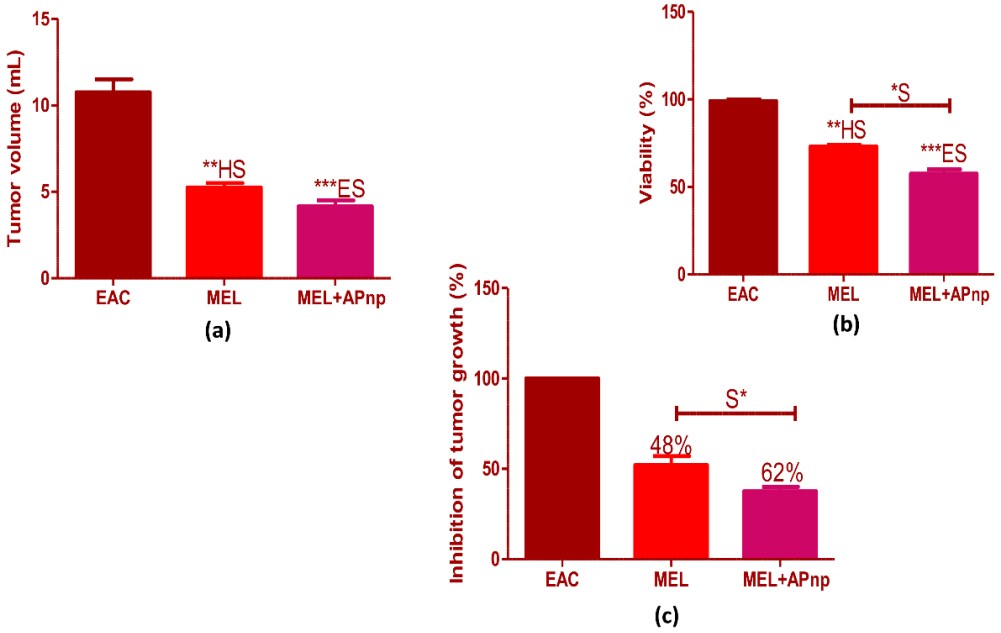

Figure 1. (a) Tumor volume (mL), **(b)** Viability percentage (%), and **(c)** Inhibition of tumor growth (%) in mice treated with MEL-alone (25 mg/kg) and combination of MEL+APnp (25 mg/kg) compared to Ehrlich ascites carcinoma (EAC) bearing mice control group (mean±SE).Significant (*S<0.05), Highly Significant (**HS<0.01), and Extremely Significant (***ES<0.001).

3.2. Antioxidants status.

The effect of MEL alone or in combination with APnp on oxidative status in the experimental mice is illustrated in Figure 2. Administration of MEL alone or MEL+ APnp *treated* mice showed a significant and highly significant increase, respectively (*P*<0.05, *P*<0.01) in the antioxidant enzyme activities of CAT and SOD, and GSH. In addition, the results in Figure 2 demonstrated a highly significant and extremely significant reduction, respectively (*P*<0.01, *P*<0.001) in the MDA and NO levels compared to the EAC control group. The present investigation indicates that the MEL, combined with APnp, showed superior antitumor and antioxidant activities in EAC-bearing mice.

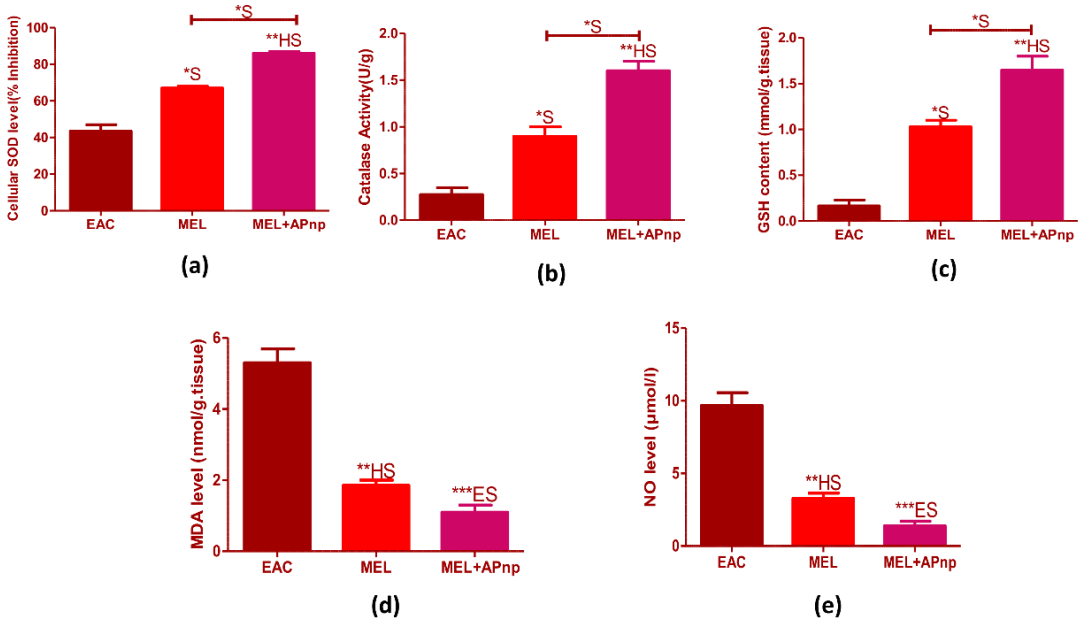

Figure 2. Antioxidant activity of MEL-alone (25 mg/kg) and combination of MEL +APnp (25 mg/kg) compared to Ehrlich ascites carcinoma (EAC) bearing mice control group (mean±SE). Significant (*S<0.05), Highly Significant (**HS<0.01), and Extremely Significant (***ES<0.001).

3.3. Histopathological observations.

Stained control EAC cells with H&E revealed normal features of cancer cells, which were characterized by irregular nuclei surrounded by basophilic cytoplasm and palpable mitotic figures. MEL-treated animals revealed slight damage in EAC cells, while MEL and APnp combination-treated animals displayed evident devastation compared to control animals, Figure 3.

EAC-inoculated mice showed clear signs of injured liver, kidney, and spleen. In the liver, there was congestion within portal veins accompanied by acute vacuolation, widened blood sinusoids, leukocytic infiltration, and activated Kupffer cells. Also, kidney specimens displayed congested areas, shrunken glomeruli, and some necrotic spaces. Spleen samples of EAC-treated mice showed poorly defined lymphoid follicles within a white pulp and increased cellularity of red pulp associated with hemosiderosis and few necrotic foci. However, the MEL-treated group revealed minor restoration in all testified organs, while combined MEL and APnp- treated animals displayed a noteworthy amelioration for all examined organs when compared to the control EAC inoculated group, Figure 3.

3.4. Ultrastructural observations.

Transmission electron microscopic image of EAC-inoculated mice illustrated in neoplastic cells with ovoid nuclei with dispersed clusters of chromatins and the cells encircled by double membrane associated with membrane blebs. Besides, mitochondrial profiles and fat droplets have existed. On the other hand, MEL demonstrated small damage, which is represented by nuclei shrinkage with the swallowed nuclear membrane and depleted mitochondria. Strikingly, MEL and APnp combination showed fragmented nuclei with condensed chromatin and apoptotic bodies. Fragmented mitochondria and lysosomal profiles were presented as well in Figure 4.

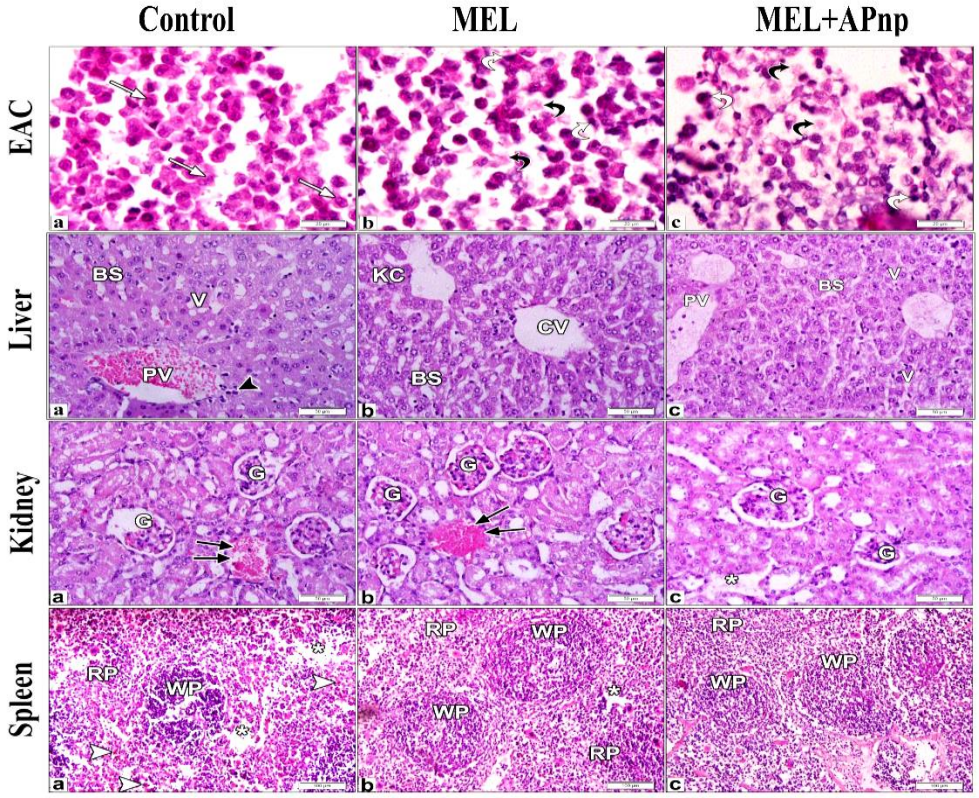

Figure 3. Microscopic image of H&E stained EAC cells **(a)** control group showing a high rate of tumor growth presented by pleomorphism, anaplasia, and increased mitotic figures. **(b)** MEL treated group represented minor damage compared to **(c)** MEL+APnp treated group, which showed moderate devastation of EAC cells. Liver sections of **(a)** the control group showed congested portal vein (PV), dilated blood sinusoids (BS), leukocytic infiltration (LI), and some vacuolation (V). **(b)** MEL treated group showed mild amelioration with activated Kupffer cells (KC), **(c)** MEL+APnp treated group showed notable restoration of hepatic structures. Kidney sections of **(a)** the control group displaying shrunken glomerular mass (G) and congested renal areas (black arrow), **(b)** MEL treated group, and **(c)** MEL+APnp treated group displaying minor to the mild restoration of renal profiles, respectively with the presence of some necrotic foci (*). Spleen sections of **(a)** the control group displaying poorly defined white pulps (WP) and congested less cellularity of the red pulp (RP) accompanied by necrotic foci (*) and hemosiderosis (white arrowheads), **(b)** MEL treated group and **(c)** MEL+APnp treated group displaying minor to mild amelioration of splenic profiles respectively representing in well-defined lymphoid follicles associated with regular red pulp.

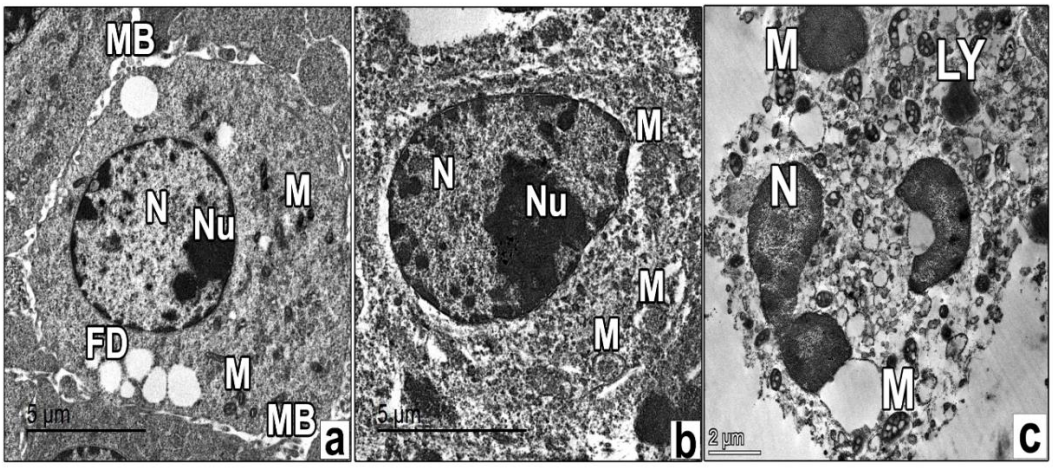

Figure 4. Transmission electron microscopic image of EAC inoculated cells **(a)** control group showing nucleus (N), with nucleolus (Nu), mitochondria (M), fat droplets (FD) and membrane blebs (MB), **(b)** MEL treated group and **(c)** MEL+APnp treated group displaying minimal to moderate damage of EAC cells, last group displayed lysosome (LY) and fragmented mitochondria.

3.5. DNA fragmentation.

The apoptosis induction caused by the combination of MEL and APnp was further investigated by assessing the DNA damage using Comet assay. Single-cell gel electrophoresis from EAC tumor cells showed comet formation in different treatment groups. Consequently, DNA damage was measured as the tail moment of DNA in the control group and the treated animal groups. In (Figure 5), mice treated with a combination of MEL and APnp exhibited a marked increase in DNA tail moment in tumor cells compared to the group treated with MEL-alone.

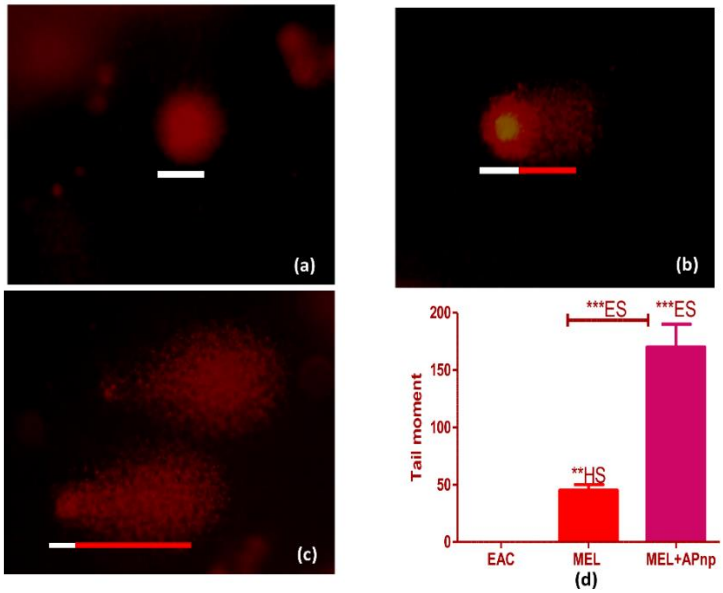

Figure 5. Evaluation of Comet assay in EAC cells. **(a)** EAC control group, **(b)** EAC treated with MEL-alone (25mg/kg), **(c)** EAC treated with a combination of (MEL+APnp 25mg/kg). White line (head, intact DNA) and the red line (tail, DNA damage). **(d)** The level of DNA damage was assessed in terms of comet tail moment. Highly Significant (**HS<0.01) and Extremely Significant (***ES<0.001).

3.6. Inhibitory effects of MEL in combination with APnp on cell growth and cell cycle.

Flow cytometry analysis was performed to demonstrate the distribution of the cell cycle. As illustrated in Figure (6a), in the MEL-treated group, a sub-G1 phase (apoptotic cells) appeared in the cell cycle distribution by 45%. Moreover, combined treatment of MEL and APnp exhibited a significant increase in the sub-G1 population (70%) compared to MEL alone.

3.7. MEL in combination with APnp modulates the IL-6/STAT3 pathway.

To explore the effect of MEL alone or in combination with APnp on the STAT3 pathway, we detected the expression of p-STAT3 in EAC cells by western blotting. The results shown in Figure (6b) suggested that a combination of MEL and APnp reduced the expression of p-STAT3, and therefore, we hypothesized that the antitumor effect of a combination of MEL and APnp was related to the STAT3 pathway. IL-6 was confirmed that it could take a promoter role in STAT3 signaling in various cells. ELISA assay was performed to set out the level of IL-6, and we found that the IL-6 levels were significantly decreased (P<0.05) in EAC cells after treatment with MEL alone, while a combination of MEL with APnp exhibited a highly significant reduction (P<0.01) when compared to control EAC group in Figure (6b,c).

3.8. MEL in combination with APnp inhibits EAC cell migration and invasion.

A combination of MEL and APnp could inhibit the activation of the IL-6/STAT3 pathway effectively. Thus, we investigated the effects of MEL alone and a combination of MEL and APnp on EAC cell invasion and metastasis. We performed a western blotting assay to detect the protein levels of MMP-9. As shown in Figure (6b), the expression of MMP-9 was significantly downregulated by a combination of MEL and AP. The observed results expressed that invasion and migration were significantly restrained by treatment with a combination of MEL and APnp.

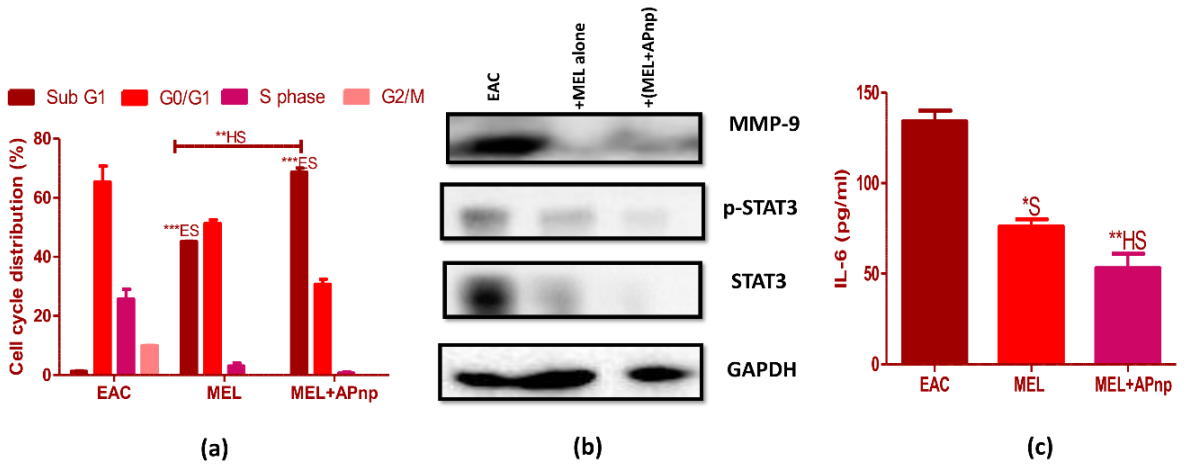

Figure 6. Antitumor effect of MEL alone (25 mg/kg) and a combination of MEL +APnp (25 mg/kg) in EAC-bearing mice on the **(a)** distribution of cell cycle by flow cytometry. (**b)** protein expression of STAT3, p-STAT3, and MMP-9 by western blot analysis. **(c)** IL-6 levels by ELISA assay.
Significant (*S<0.05), Highly Significant (**HS<0.01), and Extremely Significant (***ES<0.001).

3.9. Immunohistochemical observations.

The Ki-67 proliferation marker and Bcl-2 antiapoptotic marker were over-expressed in EAC-inoculated mice, while MEL and a combination of MEL and APnp-treated mice scored moderate to weak expression, respectively, when compared to the control group indicating less proliferation and antiapoptotic marks, Figure 7.

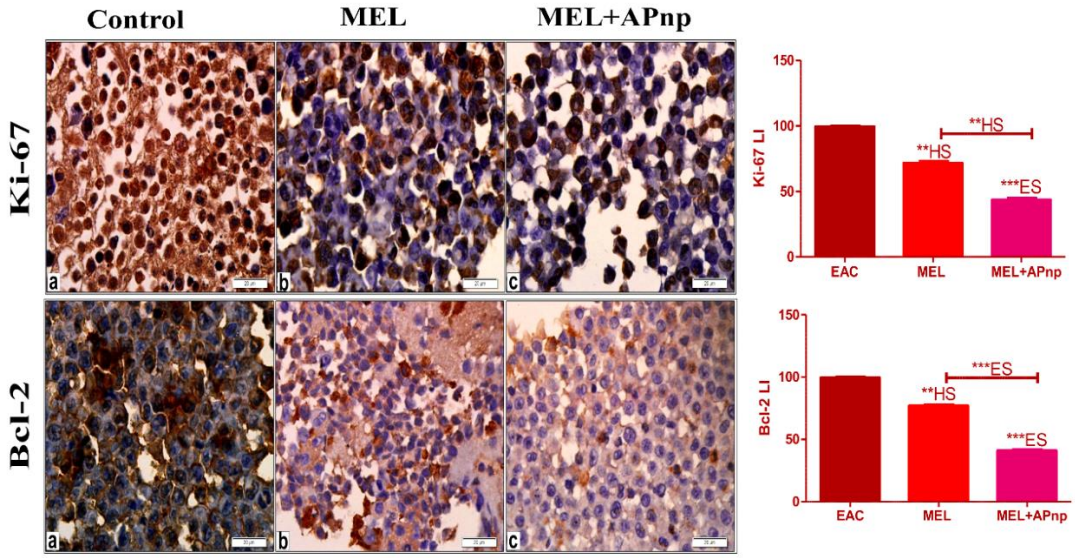

Figure 7. Immunohistochemical stain of Ki-67 and Bcl-2 representing complete positivity for both antibodies within **(a)** the control group and for **(b)** MEL treated group and **(c)** MEL+APnp treated group showing moderate to weak positivity, respectively.
Highly Significant (**HS<0.01) and Extremely Significant (***ES<0.001).

4. Discussion

Using a combination of drugs may target multiple objectives in a disease simultaneously. In cancer, using multiple anticancer agents with different mechanisms of action may also augment their therapeutic effects and treat cancer more effectively. In the present study, we aimed to explore the combined effect of melatonin and nanoformulation of ascorbyl palmitate and find a better cancer treatment strategy in the EAC-bearing mice model.

It has been reported that the administration of MEL can inhibit cancer development [41]. In addition, El-Far *et al.* reported recently that APnp efficiently inhibited tumor growth and showed promising anticancer action against EAC-bearing mice [26]. Combination therapy is an effective and promising treatment for cancer due to maximizing the therapeutic efficacy and overcoming drug resistance [42]. These two anticancer drugs have the potential to increase antitumor efficacy by suppressing tumor growth. The current study showed that the combination of MEL and APnp exhibited antitumor activity against EAC tumor cells, as evidenced by the remarkedly reduction of tumor volume and viability of EAC cells compared to MEL-alone. Furthermore, our data showed for the first time that the combination of MEL and APnp has a synergistic effect against EAC cells, which could potentiate the anticancer effect of both investigated drugs on EAC-bearing mice, thus overcoming adverse effects of high dose and low bioavailability of MEL. Therefore, combined treatment using these two active drugs can be a potent chemotherapy regimen in cancer treatment. Several studies have reported the beneficial effects of synergistic interaction between two agents in cancer therapy, which include enhancing the efficacy of the therapeutic effect, lowering the dose but increasing or maintaining the same efficacy to prevent toxicity and reducing drug resistance [43-45]

Oxidative stress is pivotal in cancer initiation and progression by enhancing tumor progression and cell proliferation; hence, antioxidant agents could be used as an essential strategy to prevent carcinogenesis [46]. Our present study found an obvious increase in the levels of MDA and NO as well as a decrease in the levels of CAT, SOD, and GSH in EAC-bearing mice; this is in agreement with another study which stated that Ehrlich tumor-induced toxicity *via* an elevation in MDA level and depletion in the SOD, CAT, and GSH levels in tumor tissue [47]. Importantly, the combination treatment of MEL and APnp can inhibit the level of MDA and NO as oxidative stress biomarkers and modulate the antioxidant status by promoting the levels of CAT, SOD, and GSH. These findings agree with other studies which detected the antioxidant activity of MEL and APnp [26,48]. Our data demonstrated that MEL combined with nanoformulation of AP could achieve superior antioxidant and anticancer through significantly inhibiting tumor growth and oxidative stress.

Herein, we aimed to highlight the implementation of melatonin either solely or coupled with APnp as a prospective strategy for cancer therapy. The histological outcomes of EAC-treated animals displayed similar characteristics to earlier research [26]. MEL and APnp combination showed signified clear damage for EAC cells when compared to MEL-only treated animals, and this supports the idea of pairing drugs together to achieve better results. MEL and APnp-treated EAC animals revealed considerable restoration of liver, kidney, and spleen tissues when compared to the control EAC inoculated group and/or MEL-treated animals; these findings were in accordance with others Batista *et al.*, who stated the possible oncostatic activity that is inhibited by melatonin [49]. Also, El-Far *et al.* reported for the first time the efficiency of APnp in fighting cancer cells [26]. In addition, Zhou and his team reported the amelioration effect of MEL in lowering hepatic injuries *via* mitochondrial

mitophagy restoration [50]. Kidney specimens of MEL and APnp-treated mice showed a much better restoration of renal profiles when compared to control or melatonin-treated groups, and this was confirmed by Bizzarri *et al.,* who investigated that melatonin enhanced the apoptotic influence of thapsigargin which considered an endoplasmic reticulum stress stimulator in kidney cancer [51]. Additionally, we previously supported the concept of ameliorative efficiency of APnp on kidney structures of EAC-inoculated mice [26]. Maleki and his colleagues supported the ability of melatonin to minimize the cytotoxicity caused by various kinds of kidney carcinogens [52]. Similarly, splenic tissues of melatonin and APnp-treated animals presented moderate restoration compared to the control group, which was in agreement with El-Far *et al.,* who highlighted similar outcomes [26]. Likewise, another study revealed the possible effect of melatonin in increasing programmed cell death in the cells of Jurkat leukemia stimulated by radiation while lowering apoptosis in control splenocytes induced by radiation [53].

Ultra-structurally, nuclei of EAC cells treated with MEL and APnp combination were clearly fractionated, and fragmented mitochondria associated with lysosomal profiles were scattered here and there; these pathological alterations were compatible with that of Grossmann *et al.* [54]. The animal group treated with MEL and APnp showed better results than that treated with melatonin, only pointing out that MEL combined with APnp exerted a powerful beating effect on neoplastic cells. Others emphasized that co-treatment of melatonin with other drug make it more efficient in diminishing cancer cells than using individual drugs [55]. Fragmented mitochondria may be attributed to melatonin impact, which was recently identified to bind with MT1 and MT2 on outer membrane receptors of mitochondria [56] and block the production of cytochrome c from the mitochondria, which consequently leading to cell death [57,58]. Recent discoveries proved the impact of melatonin on mitochondrial performance through lessening ATP manufacturing and preventing telomerase action [59]. Considering melatonin is a multifunctional antioxidant in normal cells, the synthesis of melatonin in healthy cells' mitochondria may eliminate ROS and enhance the release of antioxidant enzymes assisting in the death of unhealthy cells [60-64]. Amazingly, MEL with APnp-treated animals presented fragmented mitochondria accompanied by lysosomes referring to the mitophagy process, and this was in accordance with Twig *et al.,* who reported that frequent mitophagy might lead to apoptosis [65].

The disruption of cell cycle progression in cancer cells is considered a promising strategy to control tumor growth [66] using MEL and APnp [26,67]. Our data showed that MEL alone or a combination treatment of MEL and APnp increased the sub-G1 phase ratio in EAC cells and induced G1 phase arrest. Our present results exhibit that combination of MEL and APnp was effective in growth inhibition, deregulation of the cell cycle, and apoptosis in EAC cells.

In addition, the comet assay is an efficient method of detecting DNA damage [68,69]. We performed comet assays to measure DNA damage in EAC cells and evaluate the therapeutic effect of MEL combined with nanoformulation of AP (APnp). Our findings of the comet assay confirmed that the combination of MEL and APnp induced DNA damage in EAC cells more than in MEL alone. It has been demonstrated that DNA damage-inducing therapies are of tremendous value for cancer treatment *via* suppression of cellular proliferation [70].

Furthermore, we investigated the combined effect of MEL with APnp on the IL-6/STAT3 pathway. IL-6/STAT3 pathway can drive cell proliferation, tumor progression, invasion, and angiogenesis [26,71]; this pathway was found to be a crucial cancer drug target

[72]. The present finding demonstrated that treatment with a combination of MEL and APnp markedly inhibited the IL-6/STAT3 signaling pathway through downregulation of IL-6-induced activation of STAT3 and significant inhibition of p-STAT3 expression. Our present results suggest that the inhibition of tumor growth by the combination of MEL and APnp may be through the antiproliferative effect caused by blocking the IL-6/STAT3 pathway. Additionally, MMP-9 is a cancer biomarker that plays a critical role in cancer cell invasion and tumor metastasis [73]. In this study, the administration of a combination of MEL and nanoformulation of AP (APnp) significantly inhibited MMP-9 expression. We suggest the promising anticancer activity of MEL and APnp is attributed to the inhibition of metastasis [26,74].

Immunohistochemical studies of our new mixture revealed the most devastating effect on EAC compared to melatonin-only treated animals, illustrated in decreased scores of Ki-67 and Bcl-2 antibodies. This reflects the significant action of our combination toward EAC cells. Furthermore, it has been investigated that MEL or APnp could block the proliferation and down-regulate Ki-67 and Bcl-2 expression [26,48]. Mitochondria were responsible for managing apoptosis *via* regulating various pro-and antiapoptotic proteins like Bcl-2 and Bax through producing cytochrome c that will lead to cell death [75]. Similar findings were achieved by Xu and his team, who reported that melatonin, whether alone or paired with another drug, hindered the proliferation of EAC cells by lowering Bcl-2 and increasing Bax scores [76].

To conclude, this research shed new light on the effectiveness of a new combination of MEL and APnp in fighting EAC cells which represented significant strength in cancer treatment compared to treatment with MEL alone.

5. Conclusions

In summary, our present study demonstrates the synergistic effect of MEL and APnp, leading to enhanced antitumor efficacy in EAC tumor cells. MEL and APnp inhibited cancer cell proliferation, metastasis, and invasion. In addition, the anticancer activity of MEL and APnp is mediated by regulating several molecular targets, including IL-6/STAT3, MMP-9, Ki-67, and Bcl-2. Moreover, a combination of MEL and APnp induced DNA damage and caused mitochondrial and nuclear degeneration of EAC cells. This study strongly suggests that the combination of MEL and APnp is more effective than MEL alone in cancer treatment.

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Conflicts of Interest

The authors declare no conflict of interest.

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