Phyllanthus muellerianus and Ficus exasperata exhibit anti-proliferative and pro-apoptotic activities in human prostate cancer PC-3 cells by modulating calcium influx and activating caspases

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Abstract

Purpose

Phyllanthus muellerianus (PM) and *Ficus exasperata* (FE) are plants used against cancers. We evaluated the phytochemical profiles and *in vitro* antioxidant potentials of PM and FE, and investigate their effects on cell proliferation, intracellular calcium ($[Ca^{2+}]_i$), caspases 3/9, apoptosis, oxidative stress markers, and Bax/cytochrome C expression in PC-3 cells.

Methods

The phytochemical profiles were evaluated by liquid chromatography-mass spectrometry (LC-MS), and the antioxidant by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals scavenging method. The cells were incubated for 24 hours with 3% tween 80, paclitaxel (5 nM), PM (800 and 1200 $\mu\text{g/ml}$), and FE (800 and 1200 $\mu\text{g/ml}$). After treatments, $[Ca^{2+}]_i$, caspases 3/9, apoptosis and oxidative stress parameters were measured using colorimetric kits, while the mRNA levels of Bax and cytochrome C were quantified by RT-qPCR.

Results

Nitidine, phloridzin and linoleic acid were identified in PM, while docosane, cardanol and chlorogenic acid were revealed in FE. The *in vitro* antioxidant potential of PM was greater than that of FE. Both plants inhibited the growth of PC-3 cells in a dose-dependent manner, but significantly ($p < 0.5-0.001$) increased $[Ca^{2+}]_i$, apoptosis level, caspase 3/9 activities, reactive oxygen species production and lipid peroxidation, compared with control. Moreover, the activities of superoxide dismutase, catalase and glutathione peroxidase were significantly decreased in the cells incubated with the plant extracts, PM being the most effective. Paclitaxel, PM and FE upregulated Bax and cytochrome C genes in PC-3 cells.

Conclusion

PM and FE inhibited the growth of PC-3 cells by modulating the $[Ca^{2+}]_i$ and inducing apoptosis through Bax/Cytochrome C/Caspase 3-9 signaling pathway.

Introduction

Prostate cancer is a heterogeneous disease considered as the second leading cause of cancer-related deaths in men worldwide (Rawla 2019). The measurement of prostate-specific antigen (PSA), needle biopsy, transrectal ultrasound procedure, transrectal magnetic resonance imaging and digital rectal

examinations are commonly used to detect and localize the disease before treatment (Litwin and Tan HJ 2017; Schoots and Padhani 2020; Yang et al. 2021).

The treatment of prostate cancer is based on the stage of the disease. For instance, surgery and radiation are commonly used when the disease is localized while chemotherapy is required for metastatic disease (Litwin and Tan HJ 2017; Wisniewski et al. 2020). Chemotherapeutic agents such as paclitaxel, docetaxel, and epothilones are clinically used in the management of prostate cancer (Wang et al. 2000). Given the complexity of this disease, the modern treatment is expensive and often associated with various side effects when the drugs are taken for a long time (Asay et al. 2020). Therefore, the search for an effective and safe alternative treatment is highly needed. Previous works demonstrated the efficacy of various plants against the proliferation and invasion of prostate cancer cells. For instance, *Phyllanthus* species (*P. urinaria*, *P. niruri*, *P. watsonii* and *P. amarus*) (Tang et al. 2010; 2015), *Ficus microcarpa* (Akhtar et al. 2018), *Ficus deltoidea* (Hanafi et al. 2017) and *Ficus carica* (Rubnov et al. 2001) decrease the growth of prostate cells. *Phyllanthus muellerianus* and *Ficus exasperata* also possess a wide range of pharmacological properties.

P. muellerianus (Euphorbiaceae) is a tropical plant called “Mbolongo” in Cameroon, and traditionally used to treat many ailments such as hepatitis B and C, diabetes, infertility, gastric ulcers and cancers (Rajeshkumar et al. 2002). Many phytochemicals such as caffeic acid, chlorogenic acid, gallic acid, ellagic acid and methyl gallate have been identified in this plant (Agyare et al. 2011). Tang *et al.* (2010) demonstrated that *P. urinaria*, *P. niruri*, *P. watsonii* and *P. amarus* inhibit the growth of PC-3 and MeWo cells by modulating cell cycle and inducing apoptosis. In addition, these *Phyllanthus* species also prevent tumor metastasis and angiogenesis in PC-3 and MeWo cells (Tang et al. 2015). The mixture of *Phyllanthus amarus* and *Paramignya trimera* has shown a dose dependent anti-proliferative potential in Du145, A2780 and MCF-7 cells (Nguyen et al. 2021). We previously demonstrated that *P. muellerianus* prevents the proliferation of ovarian cysts and improves reproductive potential of rats with polycystic ovary syndrome (Ndeingang et al. 2019), but the effect of this plant on PC-3 cells is unknown.

F. exasperata (Moraceae), commonly known as sandpaper is a tropical plant widely used to treat many diseases such as arthritis, hypertension, infertility, gastric disorders and cancers (Faiyaz et al. 2012; Adekeye et al. 2020). Phytochemical analysis of different parts (barks, leaves and fruits) of *F. exasperata* revealed the presence of many compounds including cinnamoyl derivatives (caffeic acid, caffeoylquinic acid, malic acid, ferulic acid and sinapic acid), flavonoid-O-glycosides (isoquercitrin, kaempferol and rhamnosyl), flavonoid-mono-C-glycosides (apigenin-8-C-glucoside, ketorhamnosyl and apigenin) flavonoid-di-C-glycosides (luteolin-6,8-di-C-hexoside and apigenin-C-deoxyhexoside-C-hexoside), and furanocoumarins (bergapten and oxypeucedanin hydrate) (Mouho et al. 2018; Mikail et al. 2019). Moreover, seven organic acids (oxalic, aconitic, citric, tartaric, malic, quinic, fumaric) were also identified in *F. exasperata* extracts (Mouho et al. 2018). The *in vitro* antioxidant potential (Akanni et al. 2014) and antiproliferative activities of *F. exasperata* have been reported in ovarian cancer cells (Bafor et al. 2017).

Although Tang et al. (2010; 2015) and Bafor et al. (2017) have reported the beneficial effects of some *Phyllanthus* sp and *F. exasperata*, respectively on MeWo prostate cancer and A2780 ovarian cancer cells, their effects on PC-3 cells have not been investigated. We characterized *P. muellerianus* and *F. exasperata* extracts by LC-MS analysis and determined their *in vitro* antioxidant potentials by DPPH assay. With the hypothesis that the phytocomponents revealed in these plants may prevent prostate cancer, we evaluated the effects *P. muellerianus* and *F. exasperata* on cell proliferation, $[Ca^{2+}]_i$, apoptosis level, ROS production, caspase 3/9 activities, lipid peroxidation, antioxidant enzymes (SOD, CAT and GSH-Px) and mRNA levels of Bax and cytochrome C in PC-3 cells.

Materials And Methods

Collection of *P. muellerianus* and *F. exasperata*

P. muellerianus roots and the stem barks of *F. exasperata* were harvested in April 2017 in Tonga (West Cameroon). The plants were identified at the Cameroon National Herbarium (CNH) under the voucher number N° BWPV03 (for *P. muellerianus*) and N° 56091/HNC (for *F. exasperata*). The roots and stem barks were separately shade-dried for 7 days, later transformed into powder and used for preparation of the methanolic extracts of *P. muellerianus* and *F. exasperata*.

Preparation of methanolic extracts of *P. muellerianus* and *F. exasperata*

250 g of *P. muellerianus* powder were macerated in methanol (1000 ml) for 72 hours and filtered using Whatman no. 1 filter paper. The filtrate was evaporated using a rotary evaporator and 15.17 g of methanolic extract was obtained (extraction yield: 6.07%) (Ndeingang et al. 2019).

F. exasperata powder (250 g) was macerated in methanol (1000 ml) for 72 hours and filtered. The filtrate was evaporated (using a rotary evaporator) and 26.43g of methanolic extract was obtained (extraction yield: 10.57%).

LC-MS analysis of *P. muellerianus* and *F. exasperata* extracts

P. muellerianus and *F. exasperata* were analyzed as described previously (Deeh et al. 2018). Briefly, after connection of the spectrometer to an Ultimate 3000 UHPLC system, the separations were done by a Synergi MAX-RP 100A, with a H₂O (+0.1% HCOOH) (A)/acetonitrile (+0.1% HCOOH) (B) gradient (flow rate 500 µL/min, injection volume 10 µl). Samples were analyzed and identification of phyto-constituents present in *P. muellerianus* and *F. exasperata* extracts was based on the information from the spectra and data present in the literature (Oboh et al. 2014; Kumar et al. 2015; Elsayi et al. 2020).

Evaluation of *in vitro* antioxidant potential of *P. muellerianus* and *F. exasperata* by DPPH assay

The antioxidant potentials of *P. muellerianus*, *F. exasperata* and ascorbic acid (positive control) were evaluated via their scavenging activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical as reported by Koleva et al. (2002) with minor modifications. 10 µL of each concentration (400, 800, 1200 and 2000

µg/ml) of *P. muellerianus* or *F. exasperata* was added to DPPH (190 µL; 150 µM) in ethanol solution. The absorbance was recorded at 517 nm and the percentage of inhibition was estimated (Karanam et al. 2020).

Cell culture

The PC-3 human prostate cancer cell line was purchased from the National Centre for Cell Science, NCCS, Pune, India. They were cultured in 5% CO₂ and 95% air in RPMI medium supplemented with 10% FBS and 1% penicillin-streptomycin at 37 °C in a humidified incubator. The cell culture was done in an aseptic condition and the medium was changed every alternative days to avoid contamination. Every week, the cultures were split into different flasks after the cells reached at 80% confluence.

Measurement of cell viability and treatments

The effects of plant extracts on the viability of PC-3 cells were investigated by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Övey et al. 2015; Deeh et al. 2018; Nazıroğlu et al. 2019). Briefly, the cells were seeded in 96-well plates (1.5 x 10⁴ cells/well). After the cells reached good growth, cells were incubated for 24 hours with the vehicle or various concentrations (400, 800, 1200 and 2000 µg/ml) of plant extracts. In each well, 200 µl of MTT (5 mg/ml: 1X PBS) was added and incubated for 2 h. DMSO (200 µl) was added in each well and absorbance was recorded at 650 nm using Perkin Elmer multimode plate reader. All experiments were carried out in triplicates. Two moderate doses (infra IC₅₀: 800 µg/ml and supra IC₅₀: 1200 µg/ml) of plant extracts were selected and used for further studies.

Further, the cells were treated as follows: (1) control group: cells were kept in a flask without any drug; (2): tween 80: cells were incubated with 3% tween 80; (3): paclitaxel: cells were treated with paclitaxel at 5 nM; (4 and 5): *Fe* 800 and 1200 µg/ml: cells were incubated with the methanolic extract of *F. exasperata* at 800 and 1200 µg/ml, respectively; (6 and 7): *Pm* 800 and 1200 µg/ml: cells were treated with the methanolic extract of *P. muellerianus* at 800 and 1200 µg/ml, respectively. The methanolic extracts of *F. exasperata* and *P. muellerianus* were dissolved in 3% tween 80 before treatments. The dose of paclitaxel (5 nM) was chosen in a previous study (Zhang et al. 2020). At the end of the incubation period (24 hours), intracellular calcium concentration ([Ca²⁺]_i), apoptosis level, caspase 3/9 activities, oxidative stress-related markers (ROS, MDA, SOD, CAT and GSH-Px) and mRNA levels of Bax and cytochrome C were measured.

Determination of [Ca²⁺]_i

The [Ca²⁺]_i was estimated using fura-2 acetoxymethyl ester (Fura-2/AM) as described previously (Deeh et al. 2018; Gökçe et al. 2019). Briefly, after treatment with the vehicle or drugs, the cells were loaded with 4µM fura-2/AM in the dark for 45 min and washed three times with phosphate buffer. The cells were incubated for an additional 30 min and re-suspended in loading buffer. All cells incubated with the vehicle

or drugs were treated with H₂O₂ to stimulate [Ca²⁺]_i release and the fluorescence was recorded using a spectrofluorometer. [Ca²⁺]_i was estimated as described earlier (Grynkiewicz et al. 1985; Nazıroğlu et al. 2019).

Measurement of apoptosis level and caspase 3/9 activities

After treatment (24 hours) with the vehicle or drugs, the apoptosis assay was conducted using a colorimetric kit (Biocolor Ltd, Northern Ireland) following the manufacturer's instructions as described earlier (Özdemir et al. 2016). The determination of caspases 3/9 activities was done by fluorescence microscopy as previously described (Espino et al. 2010; Nazıroğlu et al. 2019). The results were expressed as fold increase over the pretreatment level (experimental/control).

Evaluation of ROS, lipid peroxidation, SOD, CAT and GSH-Px levels

ROS production was estimated using dihydrorhodamine-123 (DRH 123) as described previously (Joshi et al. 2011; Keil et al. 2011). Indeed, DRH 123 easily penetrates inside the cells and becomes fluorescent after oxidation. The intensity of fluorescence is proportional to ROS accumulation. In all samples, the intensity of fluorescence was recorded at 488 nm (excitation) and 543 nm (emission) using a professional micro plate reader (Infinite pro200).

The activities of SOD and catalase were measured as described by Shiraishi et al. (2005) and Sinha et al. (1984), respectively. Glutathione peroxidase (*GSH-Px*) activity and MDA level were measured using a detection kit according to the manufacturer's instructions.

RNA extraction and quantitative real-time polymerase chain reaction

RT-qPCR was performed to detect the expression level of Bax and Cytochrome C in PC-3 cells after treatment with the reference drug (paclitaxel) and *F. exasperata* and *P. muellerianus* extracts. The experimental conditions were the same as described previously (Olugbodi et al. 2019). Briefly, total RNA from the PC-3 cells was extracted using Qiagen RNeasy Mini Kit as defined according to the manufacturer protocol. Total RNA was reverse transcribed using the SuperScript1 VILOTM Kit and RT-qPCR was performed with SsoAdvancedTM SYBR1 and a CFX96 TouchTM devices. Gene specific primers Bax forward 5'-TTGCTTCAGGGTTTCATCCA-3', Bax reverse 5'-CAGCCTTGAGCACCAGTTTG-3' and Cytochrome C forward 5'-AGTGGCTAGAGTGGTCATTCATTTACA-3', Cytochrome C reverse 5'-TCATGATCTGAATTCTGGTGTATGAGA-3' were used for target gene amplification. To enumerate gene expression, the Ct of target gene amplification was regularised to the expression level of a housekeeping gene (GADPH forward 5'-AACTCAGGAGAGTGTTCCTCG-3', GADPH reverse 5'-TGATGGGCTTCCCGTTGATG-3'), based on the relation $R = E^{Ct_{Rpl19}/Ct_{target}}$, wherever E is the magnification potency for every primer pair (Olugbodi et al. 2019).

Statistical analysis

Data were expressed as mean $\pm$ SEM. Comparison between vehicle and drug groups was made by ANOVA one-way followed by the post hoc Tukey HSD test using STATISTICA software, version 8.0 (StatSoft, Inc., Tulsa, USA). Each experiment was repeated three times. Significance level was set at 0.05 or less.

Results

LC-MS analysis

The LC-MS chromatograms of the methanolic extracts of *P. muellerianus* (Figure 1) and *F. exasperata* (Figure 2) showed the peak areas for various components. The information from the spectra and data present in the literature allow tentative identification of nitidine (molecular weight 348.37 g/mol) at RT 1.26 min with $[M+H]^+$ at m/z 348 (Figure 2 A and B), phloridzin (molecular weight 436.4 g/mol) at RT 3.19 min with $[M+H]^+$ at m/z 435 (Figure 2 A and C) and linoleic acid (molecular weight 280.4472 g/mol) at RT 3.84 min with $[M+H]^+$ at m/z 279 (Figure 2 A and D) in the methanolic extract of *P. muellerianus*. In the methanolic extract of *F. exasperata*, docosane (molecular weight 310.6027 g/mol) at RT 2.42 min with $[M+H]^+$ at m/z 311 (Figure 3 A and B), cardanol (molecular weight 298.5 g/mol) at RT 2.95 min with $[M+H]^+$ at m/z 297 (Figure 3 A and C) and chlorogenic acid (molecular weight 354.31 g/mol) at RT 4.42 min with $[M+H]^+$ at m/z 355 (Figure 3 A and D) were identified. Other phytocomponents from these plants were not identified in the current work.

Assay of DPPH radical scavenging activity

As shown in Figure 3, the DPPH scavenging activity of methanolic extracts from *P. muellerianus* and *F. exasperata* increased ($p < 0.001$) in a dose-dependent manner, *P. muellerianus* being the most effective. Ascorbic acid significantly ($p < 0.001$) increased DPPH scavenging activity from 400 to 1200 $\mu\text{g/ml}$, but decreased ($p < 0.001$) at the highest dose (2000 $\mu\text{g/ml}$) (Figure 3).

P. muellerianus and *F. exasperata* inhibited the growth of PC-3 cells

The cell viability was significantly ($p < 0.05$ - 0.001) decreased after treatment with *P. muellerianus* (Figure 4) and *F. exasperata* (Figure 5), compared to the control or tween 80 group. *P. muellerianus* was more effective (at all doses) in inhibiting the growth of PC-3 cells than *F. exasperata* (Figures 4 and 5).

Paclitaxel, *P. muellerianus* and *F. exasperata* modulated the intracellular $[\text{Ca}^{2+}]_i$ in PC-3 cells

Paclitaxel, *P. muellerianus* and *F. exasperata* significantly increased the $[\text{Ca}^{2+}]_i$ in PC-3 cells, compared to the control or tween 80 group. The effects of plant extracts were dose-dependent. The efficacy of *P. muellerianus* was higher than that of *F. exasperata*. However, paclitaxel was more powerful than plant extracts (Figure 6A and B).

Paclitaxel, *P. muellerianus* and *F. exasperata* promoted apoptosis, ROS production and caspase 3/9 activities in PC-3 cells

As shown in Figure 7 A and B, paclitaxel, *P. muellerianus* and *F. exasperata* significantly ($p < 0.05-0.001$) increased the apoptosis level and ROS production, compared to the control or tween 80 group. The effects of plant extracts were dose-dependent, *P. muellerianus* (1200 µg/ml) being the most effective.

Caspase 3/9 activities were significantly ($p < 0.05-0.001$) elevated in the cells incubated with paclitaxel, *P. muellerianus* and *F. exasperata*, compared to the control or tween 80 group. The efficacy of *P. muellerianus* was higher than that of *F. exasperata* (Figure 8 A and B).

Overall, paclitaxel was more effective in initiating apoptosis by modulating the activation of caspases 3/9 than plant extracts (Figure 7 and 8).

Paclitaxel, *P. muellerianus* and *F. exasperata* increased lipid peroxidation and oxidative stress in PC-3 cells

The level of MDA increased significantly ($p < 0.05-0.001$) in the cells incubated with paclitaxel and plant extracts, with the highest effect observed in *P. muellerianus* (1200 µg/ml) group (Figure 9).

The activities of SOD, CAT and GSH-Px were significantly ($p < 0.05-0.001$) decreased after treatment with paclitaxel, *F. exasperata* (except SOD: *F.e* 800 µg/ml group) or *P. muellerianus*, compared to the control or tween 80 group. *P. muellerianus* was more effective in modulating MDA and SOD activity while *F. exasperata* exhibited the highest effect on CAT and GSH-Px activities (Figure 10A-C).

Paclitaxel, *P. muellerianus* and *F. exasperata* up-regulated mRNA expression of Bax and cytochrome C in PC-3 cells

Paclitaxel, *P. muellerianus* and *F. exasperata* significantly increased ($p < 0.05$) the mRNA levels of Bax and cytochrome C in PC-3 cells, compared with control and tween 80 groups. The effects of plant extracts were dose-dependent. Paclitaxel was more effective than plant extracts (Figure 11).

Discussion

Natural products are effective and reliable sources for the development of anticancer drugs with slight or no side effects (Kasala et al. 2015). Numerous studies have indicated that *Phyllanthus* sp (*P. urinaria*, *P. niruri*, *P. watsonii* and *P. amarus*) (Tang et al. 2010; 2015) and *F. exasperata* (Bafor et al. 2017), respectively inhibit the proliferation of MeWo prostate cancer and A2780 ovarian cancer cells, but there is no work on the effects of these tropical plants on the PC-3 prostate cancer cells. This work was planned to evaluate the effects *P. muellerianus* and *F. exasperata* on cell proliferation, intracellular calcium concentration ($[Ca^{2+}]_i$), apoptosis level, caspase 3/9 activities, oxidative stress related markers (ROS, MDA, SOD, CAT and GSH-Px) and mRNA levels of Bax and cytochrome C in PC-3 cells. Our results herein demonstrated that *P. muellerianus* and *F. exasperata* possess potent antioxidant property *in vitro* and exhibit an anti-proliferative property on PC-3 cells by modulating the $[Ca^{2+}]_i$ and inducing apoptosis through Bax/Cytochrome C/Caspase 3-9 signaling pathway.

The DPPH assay is used to determine the capacity of a drug to scavenge free radicals (Abotsi et al. 2010). In this work, the DPPH scavenging activity assay of the methanolic extract of *P. muellerianus* significantly increased in a dose-dependent manner, as reported by Boakye et al. (2016). This antioxidant potential could be due to the presence of the components which has the ability to quench free radicals. Indeed, in the current study, LC-MS chromatograms of *P. muellerianus* revealed the presence of nitidine, phloridzin and linoleic acid. The antioxidant properties of nitidine (Chen et al. 2012), phloridzin (Rezk et al. 2002) and linoleic acid (Fagali and Catalá 2008; Xu et al. 2020) were reported previously. We also found that the *in vitro* antioxidant activity of *F. exasperata* was lower than that of *P. muellerianus*. The antioxidant potential of *F. exasperata* could be due to the presence of antioxidant components such as docosane, cardanol and chlorogenic acid. Even low doses (30–300 µg/ml) of *F. exasperata* possess a strong DPPH scavenging activity (Abotsi et al. 2010). However, ascorbic acid used as a positive control increased the DPPH scavenging activity at low (400 µg/ml) or moderate (800 and 1200 µg/ml) dose, but decreased at high concentration (2000 µg/ml). This reduction in the antioxidant potential of ascorbic acid at high dose could be due to its toxicity (Eylar et al. 1996).

P. muellerianus and *F. exasperata* significantly decreased the viability of PC-3 cells (in a dose-dependent manner) compared with control. The capacity of *P. muellerianus* to decrease the growth of PC-3 cells corroborated the work of Tang et al. (2010) who reported that other *Phyllanthus* sp (*P. urinaria*, *P. niruri*, *P. watsonii* and *P. amarus*) inhibit the proliferation of PC-3 cells via the modulation of cell cycle and induction of apoptosis through MAPKs, PI3K/Akt, NFB, and hypoxia pathways. The anti-proliferative potential of *P. muellerianus* could be attributed to the presence of various phyto-components such as nitidine, phloridzin and linoleic acid. It has been shown that nitidine exhibited preferential cytotoxic activity against the growth of human gastric cancer cells by inhibiting STAT3 signaling pathway (Chen et al. 2012). Phloridzin (Choi 2019) and linoleic acid (Lu et al. 2010) are known to suppress tumor growth by inducing oxidant stress, blocking cyclin-dependent kinases and inducing apoptosis. However, the anti-proliferative effect of *F. exasperata* observed in the current study was previously reported on ovarian cancer cells (Bafar et al. 2017), which may be due to its contain in docosane, cardanol and chlorogenic acid. The anti-proliferative potentials of *P. muellerianus* and *F. exasperata* could be associated to their ability to modulate calcium influx in the cells.

Ca^{2+} is a second messenger involved in the regulation of cellular function (Romero-Garcia and Prado-Garcia 2019). In a pathological condition, abnormal Ca^{2+} concentration inside and outside of cell activates receptor dependent signals and promotes the production of pro-apoptotic markers, such as cytochrome c, leading to apoptosis (Bardak et al. 2017). In the present work, *P. muellerianus* and *F. exasperata* significantly increased the $[\text{Ca}^{2+}]_i$ and apoptosis level in the PC-3 cells, *P. muellerianus* being the most effective. In parallel, *Phyllanthus amarus* promotes apoptosis in cervical cancer cells by activating p53 and p21 (Paul et al. 2019). Wu et al. (2012) reported the pro-apoptotic potential of *Phyllanthus urinaria* against human osteosarcoma cancer cells through activation of Fas receptor/ligand. On the other hand, the pro-apoptotic properties of *F. exasperata* have been demonstrated in various cells including A2780 ovarian cancer cells (Bafar et al. 2017). The enhancing effects of *P.*

muellerianus and *F. exasperata* on $[Ca^{2+}]_i$ and apoptosis could be associated to their content in pro-apoptotic phyto-components. For instance nitidine (identified in the methanolic extract of *P. muellerianus*) is a compound with a powerful pro-apoptotic potential via activation of Checkpoint kinase 2 (Chk2) in human cervical cancer cells (Kwon et al. 2019). Moreover, cardanol revealed in *F. exasperata* is capable to activate apoptosis in M14 melanoma cells by up-regulating p53, cytochrome C, cleaved-caspase-3 and cleaved-PARP (Su et al. 2017). The high $[Ca^{2+}]_i$ and apoptosis level observed in the cells incubated with paclitaxel is evident, because it is a reference drug used in the treatment of various cancers including lung cancer, ovarian cancer, breast cancer, and prostate cancer (Zhu and Chen 2019). Indeed, it has been reported that the beneficial effects of paclitaxel is due to its ability to induce cell death by mobilizing extracellular calcium influx and promoting apoptosis through kinetic suppression of microtubule dynamics (Wang et al. 2000).

Although calcium signaling is an important factor of triggering the apoptosis, caspase pathways and ROS are also involved (Simon et al. 2000). Under both normal and pathologic conditions, ROS induces apoptosis by increasing the extracellular calcium and up-regulating caspase genes (Simon et al. 2000). The overproduction of ROS also causes DNA damage and negatively affects the integrity of chromosomes, associated with the activation of pro-apoptotic markers, leading to cancer cell death (Görlach et al. 2015). In the present study, *P. muellerianus* and *F. exasperata* promoted caspase 3/9 activities and ROS generation in the PC-3 cells. Similarly, other *Phyllanthus* sp (*P. urinaria*, *P. niruri*, *P. watsonii* and *P. amarus*) facilitate caspases activation and increase apoptosis in PC-3 and MeWo human cancer cells (Tang et al. 2010). Although no *in vitro* study on the effects of *F. exasperata* on ROS and caspase levels has been found in the literature, this plant has significant antiproliferative and cytotoxic properties in ovarian cancer cells (Bafar et al. 2017).

The induction of oxidative stress is an important mechanism of some anticancer drugs (Wang et al. 2000). We found in the current study that paclitaxel, *P. muellerianus* and *F. exasperata* induced oxidative stress in the PC-3 cells by significantly increasing MDA level and lowering antioxidant enzymes (SOD, TAC and GPx). The capacity of plant extracts to increase oxidative stress in PC-3 cells may be due to their stimulatory effects on the activity of xanthine oxidase (as free radicals activator). In parallel, Astaxanthin (a carotenoid compound used as dietary supplement) significantly lowered the activities of SOD, CAT and GSH-Px in the PC-3 prostate cancer cells, but had an opposite effect in the RWPE-1 prostate normal cells (Görlach et al. 2015). Thus the effects of *P. muellerianus* and *F. exasperata* on normal prostate cells are highly needed.

Bax and cytochrome C are pro-apoptotic factors. In the intrinsic pathway of apoptosis, movement of Bax into the mitochondria leads to the release of cytochrome C which activates the caspase machineries, leading to apoptosis (Green 2006; Imran et al. 2020). The significant increase in the mRNA levels of Bax and cytochrome C in PC-3 cells incubated with Paclitaxel, *P. muellerianus* and *F. exasperata* indicated that their pro-apoptotic properties is due to the activation of Bax/cytochrome C/caspase pathway. To the best of our knowledge, the current work is the first to illustrate the role of *F. exasperata* and *P. muellerianus* on the proliferation of PC-3 cells. Based on the data obtained, it could be proposed that *F. exasperata* and *P.*

P. muellerianus could act by activating caspase 8 and increasing calcium influx and ROS production, leading to oxidative stress. This may up-regulate Bax proteins and increase cytochrome C release by the mitochondria. In this condition, cytochrome C (a pro apoptotic factor) could activate caspase 3/9 machineries and apoptosis pathways, which may ultimately modulate PC-3 proliferation (Figure 12). However, more molecular works are highly needed to further clarify this mechanism.

Conclusion

P. muellerianus and *F. exasperata* are potent anti-oxidative plants with strong radical scavenging activity. These plants exhibited an anti-proliferative property on PC-3 cells by modulating the $[Ca^{2+}]_i$, and inducing apoptosis through Bax/Cytochrome C/Caspase 3-9 signaling pathway. *P. muellerianus* and *F. exasperata* may be sourced for the development of an effective therapeutic drug for the management of prostate cancer.

Declarations

Ethics approval

Not applicable

Consent to participate

Not applicable

Consent to publish

Not applicable

Authors' contributions

PBDD and VV participated in the study conception and conducted the experimental research. PBDD, MA, KA and

GK participated in the data interpretation, data analysis and statistical analysis for the variables. PBDD, NSN, PW and VV drafted the paper. PBDD and VV were responsible for the overall supervision. All co-authors read and approved the final manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

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Conflict of interest

The authors declare no conflict of interest.

Data Availability

All relevant data can be provided upon request.

Availability of data and materials

Supplementary materials are available.

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Figures

Figure 1

LC fingerprint of the methanolic extract of *P. muellerianus*. (A). B, C and D are MS spectra of compounds x (Nitidine), y (Phloridzin) and z (Linoleic acid), respectively.

Figure 2

LC fingerprint of the methanolic extract of *F. exasperata* (A). B, C and D are MS spectra of compounds a (docosane), b (cardanol) and c (chlorogenic acid), respectively.

Figure 3

DPPH free radical scavenging activity of the methanolic extracts of *P. muellerianus* and *F. exasperata*.
 Number of replicate = 3. *p<0.001: compared to dose 400 µg/ml. One-way ANOVA+Tukey HSD test.

Figure 4

***P. muellerianus* inhibited the viability of PC-3 cells.** The cells were incubated for 24 hours with various concentrations (400, 800, 1200 and 2000 µg/ml) of plant extract and cell viability was estimated as described under “Materials and methods” section. Experiments were performed in triplicate. Values are mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$: compared to control. # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$: compared to tween 80. One-way ANOVA+Tukey HSD test. P.m: *Phyllanthus muellerianus*. Two moderate

doses (infra IC₅₀: 800µg/ml and supra IC₅₀: 1200 µg/ml) were selected and used for further studies. Calibration bar = 200 µm.

Figure 5

F. exasperata inhibited the viability of PC-3 cells. The cells were incubated for 24 hours with various concentrations (400, 800, 1200 and 2000 µg/ml) of plant extract and cell viability was estimated as

described under “Materials and methods” section. Experiments were performed in triplicate. Values are mean ± SEM. *p<0.05; **p<0.01; ***p<0.001: compared to control. #p<0.05; ##p<0.01; ###p<0.001: compared to tween 80. One-way ANOVA+Tukey HSD test. F.e: *Ficus exasperata*; Two moderate doses (infra IC₅₀: 800µg/ml and supra IC₅₀: 1200 µg/ml) were selected and used for further studies. Calibration bar = 200 µm.

Figure 6

Effects of *P. muellerianus* and *F. exasperata* on $[Ca^{2+}]$ in the PC-3 cells (A and B). Values are mean $\pm$ SEM. After incubation (for 24 hours) with the vehicle, reference drug or plant extracts, the cells were exposed to CHPx to stimulate Ca^{2+} release, which was measured as described under “Materials and methods” section. * $p<0.05$; ** $p<0.01$; *** $p<0.001$: compared to control. # $p<0.05$; ## $p<0.01$; ### $p<0.001$: compared to tween 80. One-way ANOVA+Tukey HSD test. CHPx; Cumene hydroperoxide; F.e: *Ficus exasperata*; P.m: *Phyllanthus muellerianus*.

Figure 7

Effects of *P. muellerianus* and *F. exasperata* on apoptosis level (A) and ROS production (B) in the PC-3 cells. Each bar represents the mean $\pm$ SEM. Apoptosis and ROS levels were determined as described under “Materials and methods” section. * $p<0.05$; ** $p<0.01$; *** $p<0.001$: compared to control. # $p<0.05$; ## $p<0.01$; ### $p<0.001$: compared to tween 80. One-way ANOVA+Tukey HSD test. F.e: *Ficus exasperata*; P.m: *Phyllanthus muellerianus*.

Figure 8

Effects of *P. muellerianus* and *F. exasperata* on caspase 3 (A) and 9 (B) activities in the PC-3 cells. Each bar represents the mean $\pm$ SEM. Caspases 3/9 activities were determined as described under “Materials and methods” section. * $p<0.05$; ** $p<0.01$; *** $p<0.001$: compared to control. # $p<0.05$; ## $p<0.01$; ### $p<0.001$: compared to tween 80. One-way ANOVA+Tukey HSD test. F.e: *Ficus exasperata*; P.m: *Phyllanthus muellerianus*.

Figure 9

Effects of *P. muellerianus* and *F. exasperata* on MDA level in the PC-3 cells. Each bar represents the mean $\pm$ SEM. * $p<0.05$; ** $p<0.01$; *** $p<0.001$: compared to control. # $p<0.05$; ## $p<0.01$; ### $p<0.001$: compared to tween 80. One-way ANOVA+Tukey HSD test. MDA: Malondialdehyde; F.e: *Ficus exasperata*; P.m: *Phyllanthus muellerianus*.

Figure 10

Effects of *P. muellerianus* and *F. exasperata* on the activities of SOD (A), catalase (B) and Glutathione (C) in the PC-3 cells. Each bar represents the mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$: compared to control. # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$: compared to tween 80. One-way ANOVA+Tukey HSD test. SOD: *Superoxide dismutase*; CAT: *Catalase*; *GSH-Px*: *Glutathione peroxidase*; F.e: *Ficus exasperata*; P.m: *Phyllanthus muellerianus*.

Figure 11

Effects of *F. exasperata* and *P. muellerianus* on mRNA expression of Bax and cytochrome C in PC-3 cells. Gene expression levels represent the mRNA expression levels relative to control levels. Each bar represents the mean $\pm$ SEM. * $p < 0.05$: compared to control. # $p < 0.05$: compared to tween 80.

Figure 12

Proposed mechanism of action of *F. exasperata* and *P. muellerianus* on the proliferation of PC-3 cells.

FAS=APO-1: apoptosis antigen 1; ROS: reactive oxygen species; $[Ca^{2+}]_i$: intracellular calcium concentration; MDA: Malondialdehyde; SOD: *Superoxide dismutase*; CAT: Catalase; *GSH-Px*: Glutathione peroxidase; Bax: Bcl-2-associated X protein.

+ = stimulation; - = inhibition.

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