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
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 ([Ca²⁺]ᵢ), caspases 3/9, apoptosis, oxidative stress markers, and Bax/cytochrome C expression in PC-3 cells. 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 h with 3% tween 80, paclitaxel (5 nM), PM (800 and 1200 µg/ml), and FE (800 and 1200 µg/ml). After treatments, [Ca²⁺], 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. 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 [Ca²⁺], 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. PM and FE inhibited the growth of PC-3 cells by modulating the [Ca²⁺], and inducing apoptosis through Bax/Cytochrome C/Caspase 3–9 signaling pathway.

Keywords P. muellerianus · F. exasperata · Prostate cancer · Apoptosis · PC-3 cells

Abbreviations
Bax Bcl-2-associated X protein
CAT Catalase
CHPx Cumene hydroperoxide
DHR 123, Dihydrorhodamine-123
DMSO Dimethyl sulfoxide
DPPH 2,2-Diphenyl-1-picrylhydrazyl

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Introduction

As an incurable disease, cancer is still main disease for life-threatening, and in 2020 records over 19 million new cases have been diagnosed, and almost 10 million deaths are recorded across the world for the year (IARC 2000). Bioactive constituents of the plants and plant extracts (Goyal et al. 2017) as well as antioxidants (Öz et al. 2017) are welcomed for the potential therapeutic effects on several types of cancers. 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 2000). 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 cancer cells (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 species 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 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. With the hypothesis that the phytochemicals revealed in these plants may prevent prostate cancer, we evaluated the effects P. muellerianus and F. exasperata on cell proliferation, [Ca2+]i, apoptosis level, reactive oxygen species (ROS) production, caspase 3/9 activities, lipid peroxidation, antioxidant enzymes and mRNA levels of Bel-2-associated protein (Bax) and cytochrome C in PC-3 cells.
Materials and methods

Chemicals, reagents and cell culture

Roswell Park Memorial Institute medium (RPMI) medium and fetal bovine serum (FBS) were purchased from Himedia Laboratories Pvt. Ltd India. Ethylene glycol-bis(2-aminoethyl- ether)-N,N,N′,N′-tetraacetic acid (EGTA), dimethyl sulfoxide (DMSO), 3-(4,5-di-methylthiazol-2-y)l)-2,5-diphenyltetrazolium bromide (MTT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, paclitaxel and penicillin–streptomycin were obtained from Sigma (St. Louis, MO, U.S.A). Dihydrorhodamine-123 (DHR 123) and Fura-2/AM were purchased from Bachem (Bubendorf, Switzerland). Caspase substrates, cumene hydroperoxide (CHPxs) and all organic solvents were obtained from Santa Cruz (Dallas, TX, USA). Cell culture flasks were obtained from Tarsons Products (P) Ltd, India. All reagents used in this study were of analytical grade.

The PC-3 human prostate cancer cell line (Cat. No. CRL-1435) 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.

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° 56,091/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 h 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 h and filtered. The filtrate was evaporated (using a rotary evaporator) and 26.43 g 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 (Bruker, Germany) 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; Elsawi 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 (Multiskan Spectrophotometer, USA) and the percentage of inhibition was estimated (Karanam et al. 2020). Ascorbic acid is a powerful antioxidant compound widely used as a positive control in the determination of in vitro antioxidant potential by DPPH method (Brand-Williams et al. 1995; Yu et al. 2011; Neube et al. 2021).

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-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Övey et al. 2015; Deeh et al. 2018; Yazıcı et al. 2018). Briefly, the cells were seeded in 96-well plates (1.5×10⁴ cells/well). After the cells reached good growth, cells were incubated for 24 h 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 (Perkin Elmer, USA). 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 flack 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): F.e 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): P.m 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 h), 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; Gökcê et al. 2019). Briefly, after treatment with the vehicle or drugs, the cells were loaded with 4 µM 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 CHPx to stimulate [Ca²⁺]ᵢ release and the fluorescence was recorded using a spectrofluorometer (Varian Inc, Sydney, Australia). [Ca²⁺]ᵢ was estimated as described earlier (Çiğ B and Yildizhan 2020; Öz and Çelik 2022; Yildizhan et al. 2022).

**Measurement of apoptosis level and caspase 3/9 activities**

After treatment (24 h) 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 using a micro plate reader (Infinite pro200; Tecan Group Ltd., Männedorf, Switzerland) as previously described (Espino et al. 2010; Naziroğlu 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; Tecan Group Ltd., Männedorf, Switzerland) (Uğuz et al. 2015).

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 Super-Script1 VILOTM Kit and RT-qPCR was performed with SsoAdvancedTM SYBR1 and a CFX96 TouchTM devices. Gene specific primers Bax forward 5′-TGCTTCAAGGT TTCATCCA-3′, Bax reverse 5′-CAGCCTTGAGCACA GTTTG-3′ and Cytochrome C forward 5′-AGTGGCTAG AGTGGTCATTCAATTTACA-3′, Cytochrome C reverse 5′-TCATGATCTGAATCTGGTGTGAGA-3′ were used for target gene amplification. To enumerate gene expression, the Ct of target gene amplification was normalised to the expression level of a housekeeping gene (GADPH forward 5′-AACCTCAAGAGAGTGTTCGTC-3′, GADPH reverse 5′-TGATGGGGTTCCCGTGTAG-3′), based on the relation R = ECtRpl19/ECttarget, wherever E is the magnification potency for every primer pair (Olugbodi et al. 2019).

**Statistical analysis**

Data were expressed as mean ± 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 (Fig. 1) and F. exasperata (Fig. 2) showed the
Fig. 1 LC fingerprint of the methanolic extract of \textit{P. muellerianus}. A, B, C and D are MS spectra of compounds x (Nitidine), y (Phloridzin) and z (Linoleic acid), respectively.

Fig. 2 LC fingerprint of the methanolic extract of \textit{F. exasperata} (A). (B), (C) and (D) are MS spectra of compounds a (docosane), b (cardanol) and c (chlorogenic acid), respectively.
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 (Fig. 2A and B), phloridzin (molecular weight 436.4 g/mol) at RT 3.19 min with [M + H]+ at m/z 435 (Fig. 2A and C) and linoleic acid (molecular weight 280.4472 g/mol) at RT 3.84 min with [M + H]+ at m/z 279 (Fig. 2A 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 (Fig. 3A and B), cardanol (molecular weight 298.5 g/mol) at RT 2.95 min with [M + H]+ at m/z 297 (Fig. 3A and C) and chlorogenic acid (molecular weight 354.31 g/mol) at RT 4.42 min with [M + H]+ at m/z 355 (Fig. 3A 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 Fig. 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 µg/mL, but decreased (p < 0.001) at the highest dose (2000 µg/mL) (Fig. 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_ (Fig. 4A) and

![Graph](image-url)
**F. exasperata** (Fig. 4B), 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* (Figs. 4A and B).

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

Paclitaxel, *P. muellerianus* and *F. exasperata* significantly increased the \([\text{Ca}^{2+}]\), 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 (Fig. 5A and B).

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

As shown in Fig. 6A 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* (Fig. 6C and D).

Overall, paclitaxel was more effective in initiating apoptosis by modulating the activation of caspases 3/9 than plant extracts.

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 (Fig. 7A).

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
Fig. 6 Effects of *P. muellerianus* and *F. exasperata* on apoptosis level (A), ROS production (B), caspase 3 (C) and 9 (D) in the PC-3 cells. Each bar represents the mean±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*

Fig. 7 Effects of *P. muellerianus* and *F. exasperata* on MDA (A), SOD (B), catalase (C) and Glutathione (D) levels in the PC-3 cells. Each bar represents the 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. MDA: Malondialdehyde; F.e: *Ficus exasperata*; P.m: *Phyllanthus muellerianus*
SOD activity while *F. exasperata* exhibited the highest effect on CAT and GSH-Px activities (Fig. 7B-D).

**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 (Fig. 8).

**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 ([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 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 [Ca²⁺]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 (Eyilar 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 (Bafor 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.

Ca²⁺ is a second messenger involved in the regulation of cellular function (Romero-Garcia and Prado-Garcia 2019). In a pathological condition, abnormal Ca²⁺ 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 (Nazıroğlu et al. 2020). In the current study, CHPx was used to stimulate [Ca²⁺]ᵢ release. It should be noted that the action of CHPx is not specific because it opens many calcium channels that are activated due to oxidative stress. In the present work, P. muellerianus and F. exasperata significantly increased the [Ca²⁺]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 (Bafor et al. 2017). The enhancing effects of P. muellerianus and F. exasperata on [Ca²⁺]ᵢ and apoptosis could be associate to their contain 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 [Ca²⁺]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 (Bafor 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. urinaria.
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 (Görlich 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 partway 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 pathray. 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. muellerianus could act by 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 (Fig. 9). 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.

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**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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**Data Availability** All relevant data can be provided upon request.

**Declarations**

**Ethical approval** Not applicable.

**Consent to participate** Not applicable.

**Consent to publish** Not applicable.

**Conflicts of interest** On behalf of all authors, the corresponding author states that there is no conflict of interest.

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