



## Antiproliferative effect of polyphenols and sterols of virgin argan oil on human prostate cancer cell lines

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### Abstract

**Background:** The aim of our study has to evaluate the antiproliferative effect of polyphenols and sterols extracted from the virgin argan oil on three human prostatic cell lines (DU145, LNCaP, and PC3). **Methods:** Cytotoxicity, anti-proliferative effects and nuclear morphological changes of cells were analyzed after treatment with sterols and polyphenols. The results were compared to 2-methoxyestradiol (2ME<sub>2</sub>) as positive control. **Results:** Polyphenols and sterols of virgin argan oil and 2ME<sub>2</sub> exhibited a dose-response cytotoxic effect and antiproliferative action on the three tested cell lines. The antiproliferative effect of polyphenols was similar for the DU145 and LNCaP cell lines; the GI<sub>50</sub> (defined as the concentration inhibiting growth by 50% in comparison with the control) was respectively 73 and 70 μg/ml. The antiproliferative effect of sterols was 46 and 60 μg/ml as GI<sub>50</sub> for the DU145 and LNCaP cell lines. For the PC3 cell line, the best antiproliferative effect was obtained by argan sterols with GI<sub>50</sub> = 43 μg/ml. On the other hand, the nuclear morphology analyses have shown an increased proportion of pro-apoptotic nuclei in LNCaP cell treated with IC<sub>50</sub> of polyphenols or sterols compared to control cells. Our results show for the first time the antiproliferative and pro-apoptotic effects of polyphenols and sterols extracted from virgin argan oil and confirm the antiproliferative and pro-apoptotic effects of 2ME<sub>2</sub> on prostate cancer cell lines. **Conclusion:** These data suggest that argan oil may be interesting in the development of new strategies for prostate cancer prevention.

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**Keywords:** Virgin argan oil; Sterols; Polyphenols; 2-Methoxyestradiol; Prostate cancer; Antiproliferative effect; Apoptosis; Low-density lipoprotein isolated (LDL)

### 1. Introduction

Prostate cancer (PC) is the second leading cause of cancer in males in the United States [1]. Evidence of the role of the genetic factors in the development of PC is underscored by the association of several genes (e.g., HPC1, PCAP, HPCX, CAPB, and 16q23) with hereditary PC (HPC) [2–4]. Racial and geographic differences in the incidence of PC strongly suggest a genetic component to this disease. The incidence of PC worldwide is higher in men from industrialized western and northern European countries than in Asian men [5], suggesting that factors such as diet may play a role in the

pathogenesis of this disease. Thus, the epidemiology of PC indicates that it is complex and multifactorial involving both genetic and environmental factors. The protective effects and/or therapeutic benefits of various dietary substances are only recently emerging [6–10]. Moreover, numerous epidemiological studies [11–13], case-control studies [14,15], and some prospective randomized trials [16,17] have defined the relationship between diet and the development of the PC. In fact, several studies [18–21] have identified various dietary substances with antioxidant properties and inhibitory effect on the development and/or progression of PC. Many of these substances (e.g., carotenoids and lycopenes, retinoids, Vitamin A, C and E, and phenol-containing dietary substances) have been suggested to prevent PC [22–25].

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Virgin argan oil is harvested from the fruits of the argan tree (*Argania spinosa*). Production of argan oil is endemic to Southwest Morocco, where it plays major ecologic and socio-economic roles [26,27]. This oil is known by its rich source of linoleic and oleic acids respectively 37% and 45%, and minor compounds (tocopherols, polyphenols, sterols, carotenoids, xanthophyls and squalene) [28]. The unsaponifiable fraction is very abundant in these compounds and have a powerful antioxidant effects as proved on low-density lipoprotein isolated (LDL) from human plasma [29]. However, in contrast to other seasoning oils, there are just few studies describing the benefit of the argan oil in preventing diseases. Recently, we have demonstrated an antiproliferative effect of argan tocopherols and saponins argan cake press on prostate cancer cell lines [30]. In this study, we have investigated the effects of polyphenols and sterols extracted from virgin argan oil, on cell viability and proliferation of three human prostatic cell lines (PC3, DU145 and LNCaP). This may help us to better understand the beneficial use of argan oil in prostate cancer prevention. The results are compared to 2-methoxyestradiol (2ME<sub>2</sub>) as antiproliferative drug candidate [31].

## 2. Materials and methods

### 2.1. Cell lines

Three human prostatic cell lines (two hormone-independent (DU145 and PC3) and one hormone-dependent (LNCaP) human prostate cell lines) were grown as monolayers in RPMI 1640 medium (Invitrogen, Cergy Pontoise, France) supplemented with 5% fetal bovine serum (FBS, Invitrogen) and 2 mM L-glutamine (Invitrogen), at 37 °C with 5% CO<sub>2</sub> and 95% humidity. The human prostate cancer cell lines LNCaP, DU145 and PC3 were purchased from the American Type Culture Collection.

### 2.2. Argan compounds extraction

The virgin argan oil used in this work was extracted by a cold pressing process and its chemical composition is listed in Table 1 [28]. The fruit is provided from the Essaouira area in the southwest of Morocco. Argan oil was used in its rough state, without any preliminary processing. In order to investigate the antiproliferative effect of this oil, we analyzed the unsaponifiable fraction that represents 1% of the total mass of the oil, for which we extracted the sterols compounds. The sterols were separately obtained after extraction of the unsaponifiable fraction of virgin argan oil, which was obtained by the hexane extraction method. Using the high performance liquid chromatography technique (HPLC), the purified fraction of sterols was reconstituted in chloroform [28] while the polyphenols were isolated by the methanol

Table 1

Composition of virgin argan oil khallouki et al. [28]

Fatty acid	%
C16:0	13.4
C18:0	5.1
C18:1 <i>n</i> – 9	44.8
C18:2 <i>n</i> – 6	35.7
C18:3 <i>n</i> – 3	0.1
Sterols	mg/100 g oil
Schottenol	142
Spinasterol	115
Stigmasta-8,22-dien-3β-ol	9
Others	29
Tocopherols	mg/kg oil
α	35
δ	122
γ	480
Phenolic compounds	μg/kg oil
Vanilic acid	67
Syringic acid	37
Ferulic acid	3147
Tyrosol	12

extraction (MeOH) method described by Owen et al. (2000) [32].

### 2.3. Steroid

The 2-methoxyestradiol was provided by Steraloids Laboratory (Wilton, NH 03086, USA). A stock solution was prepared in dimethyl sulfoxide (DMSO) (Sigma, Saint-Quentin Fallavier, France) and stored at –20 °C. The maximal final concentration of DMSO was 1 μl/ml (0.1%, v/v).

The 2ME<sub>2</sub> is synthesized in vitro by hydroxylation at the 2-position of estradiol and subsequent *O*-methylation and catechol-*O*-methyltransferase (COMT) [33,34].

### 2.4. Methyl thiazolyl tetrazolium (MTT) assay

PC3, DU145 and LNCaP cell lines were seeded on 96-well plates at density of 10,000 cells/well in a total volume of 100 μl. Cells were incubated for 24 h prior to addition of polyphenols, sterols and 2ME<sub>2</sub>. After 24 h, the medium was changed by another, which contains seven different concentrations between 1 and 100 μg/ml. The amounts of solvent DMSO used to dissolve sterols and polyphenols were the same as the control plate (medium plate).

Living cells were counted at 48 h using the MTT method [35]. Briefly, cells were incubated for 2 h at 37 °C with 1 mg/ml MTT (Sigma). MTT was then discarded and replaced by isopropanol (organic solvent). Optical density (OD) was measured on an EL800 universal microplate reader (Bio-Tek instruments, Winooski, VT, USA) at a wavelength of 550 nm. IC<sub>50</sub> was defined as the concentra-

tion entailing a 50% reduction of OD in comparison with the control.

### 2.5. Tritiated thymidine incorporation ( $^3\text{HdThd}$ )

PC3, DU145 and LNCaP cell lines were cultured on 24-well plates at density of 20.000 cells/well in a total volume of 1 ml. After plating in 24-well tissue culture plates (Becton Dickinson) and 24 h incubation, cells were treated with polyphenols, sterols and 2ME<sub>2</sub> for 24 h at different concentrations between 1 and 100  $\mu\text{g/ml}$ . A 2 h pulse of 2  $\mu\text{Ci/ml}$   $^3\text{H}$  thymidine was then applied to the cells. Thereafter, cells were incubated for 10 min at 4 °C in 10% trichloroacetic acid (TCA), washed three times with phosphate buffered saline (PBS) pH 7.4 and lyzed in 200  $\mu\text{l}$  of 0.2 M NaOH/1% SDS solution. Radioactivity incorporated in the cells was measured by scintillation counting for 1 min on Beckman LS 6000SC scintillation counter (Beckman Coulter, Roissy, France). The GI<sub>50</sub> was

defined as the concentration inhibiting growth by 50% in comparison with the control.

### 2.6. Nuclear morphological changes

LNCaP Cells grown on glass lab-tek chamber slides (Nalge Nunc, Naperville, IL) were exposed to IC<sub>50</sub> of polyphenols, sterols and 2ME<sub>2</sub> for 24 h. Nuclear morphological changes were observed by fluorescent microscopy after 10 min staining with 0.1  $\mu\text{g/ml}$  Hoescht 33342 dye (Molecular Probes, Eugene, OR) diluted in PBS. The criteria used to identify pro-apoptosis included nuclear shrinkage and chromatin condensation.

### 2.7. Statistics

Statistical analysis was performed using statview software (SAS Institute, Inc., Cary, NC, USA). The results are reported as mean (SD).

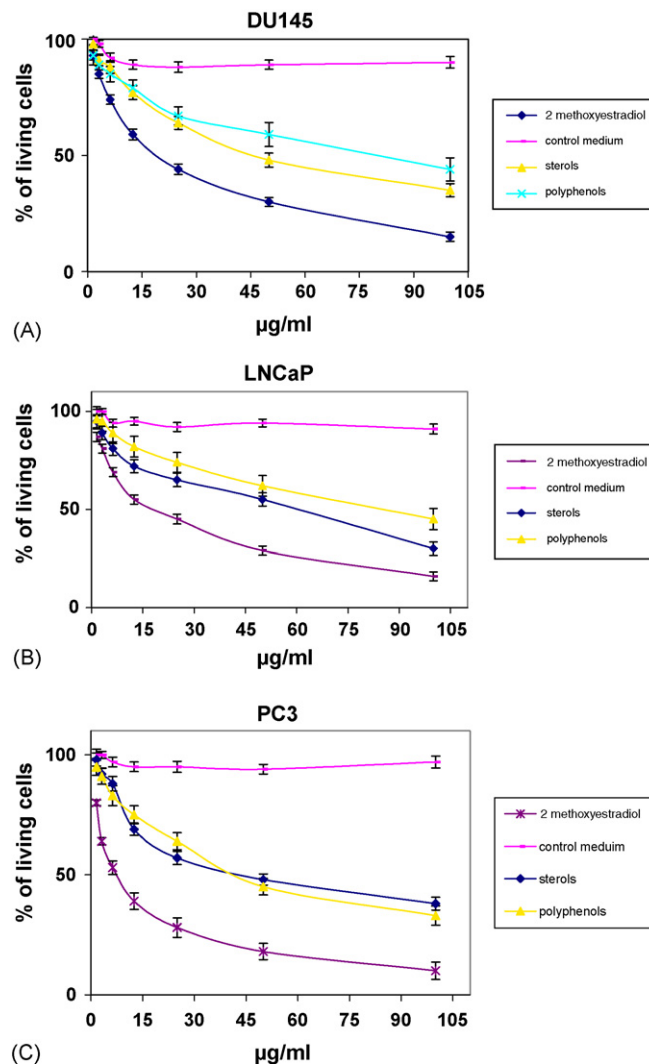


Fig. 1. Effect of polyphenols and sterols extracted from argan oil on cell proliferation compared to 2-methoxyestradiol (2ME<sub>2</sub>) as positive control. After 24 h incubation, cell proliferation of (A) DU145, (B) LNCaP and (C) PC3 cell lines was assessed by  $^3\text{H}$  thymidine incorporation. The percentage of living cells was determined in comparison with control medium used as negative control, which contains corresponding concentrations of DMSO.

### 3. Results

#### 3.1. Relative cytotoxicity of polyphenols, sterols and 2-methoxyestradiol

The relative cytotoxic activity of polyphenols, sterols and 2ME<sub>2</sub> on human tumor cells was measured by means of MTT assay in human cancer cell including the DU145, LNCaP and PC3 lines. Table 2 summarizes the means IC<sub>50</sub> values for polyphenols, sterols and 2ME<sub>2</sub>. The data indicated that polyphenols, sterols and 2ME<sub>2</sub> inhibited the growth of the three human tumor cells after 48 h. For the DU145 and LNCaP cell lines, the most cytotoxicity effect was obtained with the sterols fraction at IC<sub>50</sub> = 25.07 and 75.03 μg/ml, respectively. Whereas the polyphenols fraction showed the best antiproliferative effect on the PC3 cell line, to an IC<sub>50</sub> of 50.14 μg/ml.

#### 3.2. Polyphenols, sterols, and 2-methoxyestradiol inhibit cell proliferation

Polyphenols, sterols and 2ME<sub>2</sub> showed a dose-dependant antiproliferative effect on DU145, LNCaP and PC3 cell lines (Fig. 1). The calculated GI<sub>50</sub> at 24 h on DU145, LNCaP and PC3 cell lines were respectively 25, 75, 70 μg/ml for sterols, 75, 100, 50 μg/ml for polyphenols and 12, 11, 6 μg/ml for 2ME<sub>2</sub>. Proliferation in

Table 2

Relative cytotoxicity of polyphenols and sterols extracted from argan oil and 2-methoxyestradiol (2ME<sub>2</sub> used as the positive control), in three human cancer cell lines (measured after 48 h)

Cell lines	Sterols	Polyphenols	2ME <sub>2</sub>
IC <sub>50</sub> (μg/ml)			
DU145	25.07 ± 2.74	75.23 ± 4.14	20.11 ± 1.84
LNCaP	75.03 ± 3.41	99.98 ± 5.01	15.63 ± .32
PC3	69.77 ± 2.74	50.14 ± 3.56	6.16 ± 1.17

IC<sub>50</sub>, the concentration that inhibit growth of cell by 50% vs. control cells (control medium used as negative control). Data are presented as the mean ± S.D. of three separated experiments on each cell line.

the three cell lines was more inhibited by 2ME<sub>2</sub> than the argan compounds.

#### 3.3. Polyphenols, sterols, and 2-methoxyestradiol induce changes in cell morphology

Morphological analysis of cells treated with sterols, polyphenols of virgin argan oil and 2ME<sub>2</sub> for 24 h showed the presence of cells in mitosis in each cell lines. Moreover, treated cells exhibited a more heterogeneous morphology compared to control (Fig. 2). The percentage of apoptosis at 24 h on LNCaP cell line were respectively 7% for sterols, 5.5% for polyphenols and 8.5% for 2ME<sub>2</sub>. Apoptotic effect of 2ME<sub>2</sub> on LNCaP cell line was greater than the argan oil compounds.

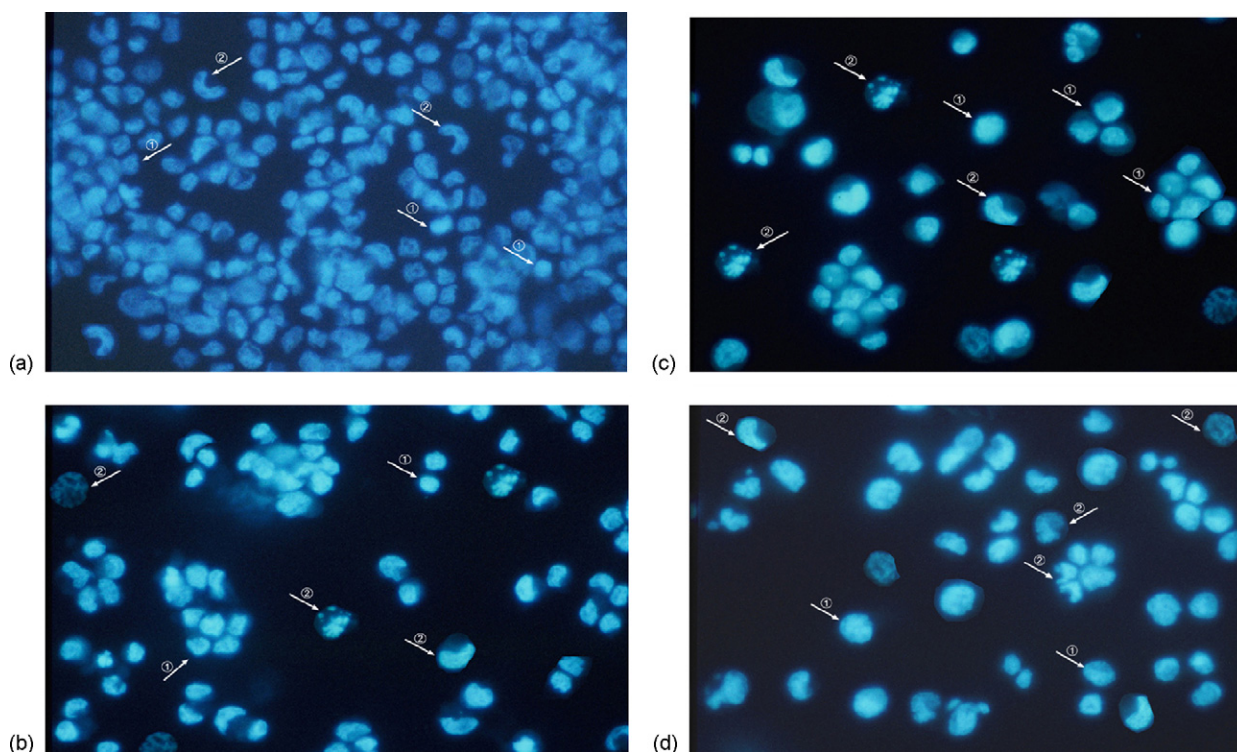


Fig. 2. Pro-apoptotic effect of Polyphenols, sterols of argan oil and 2-methoxyestradiol on LNCaP cell morphology 24 hr after treatment. Cells observed by light microscopy (40×) in the presence of IC<sub>50</sub> of each compound. (a) Control medium (negative control), (b) polyphenols, (c) sterols, and (d) 2-methoxyestradiol (positive control): (1) normal nuclear morphology; (2) apoptotic nuclear morphology.

#### 4. Discussion

Natural antioxidants in vegetable food such as tea, olive oil and wine are believed to reduce the risk of cancer [19,24,36]. Evidence supporting this hypothesis is based on epidemiological observations, animal's case studies and cell culture experiments [17,21]. Argan oil compounds are also an important dietary source of antioxidants [28]. It has been demonstrated that some argan compounds such as tocopherols and saponins have an antioxidative, antiproliferative and anti-inflammatory properties [29,30,37,38]. To our knowledge, no study of the effects of sterols and polyphenols extracted from the virgin argan oil on prostate cancer has been reported to date.

In the present study, we evaluated the effect of polyphenols and sterols obtained from virgin argan oil in order to investigate the possible prevention of prostate cancer by studying proliferation in two hormone-independent (DU145 and PC3) and in one hormone-dependent (LNCaP) human prostate cell lines. Our data indicate that the polyphenols of argan oil exerted an inhibitory effect on the proliferation of DU145, LNCaP and PC3 cell lines ( $GI_{50} = 75, 100, 50 \mu\text{g/ml}$ , respectively). The PC3 was more sensitive than the two other cell lines. These results are in agreement with several *in vitro* studies [24,39–42]. According to Hiipakka et al. (2002) [39], the tea polyphenols could influence some biochemical processes such as inhibition of an enzyme strongly expressed in prostate cancer, ornithin decarboxylase. Also Soya polyphenols could inhibit the autophosphorylation of EGF receptor (epithelial growth factor) which has a tyrosine-kinase activity, normally activated by the EGF [40]. These possible mechanisms show the interest to elucidate the exact mode of action of argan polyphenols and to evaluate their effectiveness in the treatment of prostate cancer in man. Taken together, our findings are promising for the future use of argan oil in patients developing prostate cancer.

Moreover, our results showed that sterols of argan oil produced a time and concentration-dependent decrease in the number of viable cells in all three tested cell lines. DU145 was the most sensitive with only  $\cong 30\%$  viable cells after 24 h treatment with  $25 \mu\text{g/ml}$ . Several studies have demonstrated a significant inverse association between the dietary intake of phytosterols and the risk of prostate cancer [43–45]. Indeed, Von Holtz et al. (1998) observed on prostate cancer cell lines, treated by the phytosterols a reduction of 24% of the growth and a multiplication by four the rate of apoptosis, compared to the same cells treated by the cholesterol. In addition, it has been proposed that the  $\beta$ -sitosterol is effective in the treatment of benign prostatic hypertrophy [46–48]. The major sterols identified in argan oil were schottenol and spinasterol. These sterols are very rare in vegetable oils. There are few studies reporting the anticarcinogenic effect of schottenol and spinasterol. So, it will be interesting to investigate separately these argan sterols in order to clarify the role of each one in cancer prostate prevention.

Before drawing final conclusions about the mechanisms of the anticancer effects of polyphenols and sterols extracted from argan oil, further investigations concerning the cell proliferation and apoptosis would be necessary. Our study suggests for the first time that argan oil compounds could play a role in developing new strategies for the prevention and treatment of prostate cancer. Various studies showed that tocopherols, phytosterols, and polyphenols exert beneficial effects. Knowing the interesting chemical composition of argan oil rich in unsaturated fatty acids and minor compounds, we can consider the possible synergistic effects of these compounds that would be more beneficial than the use of each one.

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