Effects of *Acorus calamus* plant extract on prostate cancer cell culture

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Acorus calamus bitki ekstraktının prostat kanser hücre kültürü üzerine etkileri

Abstract: In western countries, prostate cancer is the most frequently diagnosed cancer and the second most common cause of death from cancer in men. Vascular endothelial growth factor-A (VEGF-A), thought to be the single most important angiogenic factor in prostat cancer. Poly-(ADP-ribose) polymerase (PARP) involved in apoptotic process and cleavage of PARP serves as a marker of cells undergoing apoptosis. *Acorus calamus* have long been considered to have anti-carcinogenic and medicinal properties especially in Asia. We examined whether ethanolic extract of *A. calamus* root affects the survival of prostate cancer LNCaP cells and induces apoptosis and angiogenesis of these cells *in vitro*. Cells were incubated during 24 and 48 hours with various doses of extract. Extract with these concentrations reduced the number of LNCaP living cells up to 44 % as compared to the control at dose and time dependent manner at 24 and 48 hours. Significantly alterations were observed at cleaved PARP, VEGF-A protein and gene expression amounts after 24 and 48 hours. The present study reveals the possibility that ethanolic extract of *A. calamus* root posseses a dose and time dependent anticancer, apoptotic and anti-angiogenic properties.

Key words: LNCaP, *Acorus calamus*, anti-cancer, anti-angiogenic, apoptotic

Özet: Batılı ülkelerde erkekler arasında prostat kanseri en sık tanısı koulan kanser türüdür ve erkeklerde kanser nedeniyle ölümde ikinci sırada yer almaktadır. Prostat kanserinde vasküler endotelyal büyüme faktör-A'nın (VEGF-A) tek başına en önemli anjiogenik faktör olduğu düşünülmektedir. Apoptotik süreçte yer alan poli-(ADP-riboz) polimeraz'ın (PARP) yıkılımı apoptotik süreç markırı olarak kullanılmaktadır. *Acorus calamus*'un anti-kanserojen etkisi ile birlikte çeşitli tıbbi özelliklere sahip olduğu uzun yıllardır özellikle Asya'da kabul edilmektedir. Çalışmamızda *A. calamus* kökünün etanolik ekstraktının insan prostat kanser LNCaP hücre dizisinde hücre çoğalmasına, anjiogeneze ve apoptozise olası etkileri *in vitro* ortamda çalışıldı. Hücreler 24 ve 48 saat boyunca çeşitli ekstrakt konsantrasyonlarıyla muamele edildi. Bu konsantrasyonlardaki ekstraktlar 24 ve 48 saatte LNCaP hücre yaşayabilirliğini kontrolle karşılaştırıldığında doza ve zamana bağlı olarak %44'lere kadar azalttı. Bölünmüş PARP, VEGF-A proteini ve gen ekspresyon miktarlarında 24 ve 48 saat sonra belirgin değişiklikler gözlemlendi. Bu çalışma *A.calamus* kökünün etanolik ekstraktının zaman ve doz bağımlı olarak güçlü bir anti-kanser, anti-anjiogenik ve apoptotik etkileri olabileceğini ortaya koymaktadır.

Anahtar Kelimeler: LNCaP, *Acorus calamus*, anti-kanser, anti-anjiogenik, apoptotik

1. Introduction

Traditional medicine (TM) is very popular not only in Asia or Africa, but also in Europe and in the United States (Zuba and Byrska, 2012). Historically, the majority of new medicines are either derived from natural products or from natural products (Jesse and Verdaras, 2009). In recent years, the use of traditional medicine has increased significantly in developed and developing countries. The use of *Acorus calamus* L. plant, in addition to foodstuffs in India and Far East countries, for medical purposes is based on a very long history. In studies conducted, these plants have been reported to have characteristics such as anticancer (Pandit et al., 2011; Bisht et al., 2011), anti-inflammatory (Kim et al., 2009), anti-microbial (Devi and Ganjewala, 2009), anti-inflammatory (Joshi, 2016), neuroprotective (Shukla et al., 2006), antioxidant (Acuna et al., 2002) and antiasthmatic effects (Shah and Gilani, 2010). Different parts of the plant showed the presence of large number of phenyl propanoids, sesquiterpenes and monoterpenes as well as xanthone glycosides, flavones, lignans, steroids and inorganic constituents (Raja et al., 2009). The most important ingredients of *A. calamus* are

asarones, that is alpha- and beta-asarone (Zuba and Byrska, 2012).

Angiogenesis is the physiological processes through which new blood vessels form from pre-existing vessels. It leads to tumor expression of pro-angiogenic factors such as angiogenin, vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), TGF-β, etc. and resulted in an increased tumor vascularization. These factors are reported to promote proliferation of new vascular structures from blood vessels. Inhibition of angiogenesis leads to suppression of "angiogenic switching" which prevents activation of pro-angiogenic factors and subsequently the tumor progression halts (Mahapatra et al., 2015). Angiogenesis has become an attractive target for anticancer chemotherapy due to its important role in the progression of solid tumors (Wei et al., 2011). It has been reported that tumor growth and metastasis are angiogenesis dependent processes and that inhibition of angiogenesis may be a strategy for the prevention of disease progression (Folkman, 1971). Tumor cells produce pro-angiogenic growth factors. Among these factors, vascular endothelial growth factor (VEGF) is the most

important and accelerates all stages of angiogenesis and shows pro-angiogenic properties. It has been shown that VEGF is overexpressed in high amounts in various tumors. Therefore, VEGF-targeted approaches to tumor angiogenesis are of great importance (Gerald, 2012).

Apoptosis, also known as programmed cell death or cell suicide, allows the death of the cell at the end of a particular series of molecular manipulations and is also required for the maintenance of embryonic development and adult tissue development. Apoptosis is characterized by unique phenotypic changes involving cell shrinkage, chromatin condensation, plasma membrane blistering and the formation of apoptotic bodies, and walking is an energy-dependent process (Bohm and Schil, 2003). A nuclear enzyme, poly (ADP-ribose) polymerase (PARP) plays an important role in the repair of DNA yarn breaks. This enzyme binds by recognizing DNA strand breaks that occur with oxidative damage or other causes and facilitates protection of the cell from DNA damage. The destruction of PARP, which is also a target of caspase-3, is one of the processes of apoptotic mechanism. (Brock et al., 2004).

In this study, we investigated the effects of *A. calamus* extracts on cell proliferation, apoptosis and angiogenesis in human prostate carcinoma LNCaP cells..

2. Materials and Method

2.1. Cell line and culture conditions

The LNCaP cell line was obtained from the Yeditepe University, Faculty of Medicine, Department of Physiology (Istanbul, Turkey). The cell line was cultured in Roswell Park Memorial Institute medium (RPMI) 1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin G and 100 mg/ml streptomycin and kept at 37 °C in a humidified incubator with an atmosphere of 5% CO_2 and 95% air.

2.2. Preparation of ethanolic extract of *A. calamus*

Plant processing was done in the Anadolu University Medicinal Plants, Drugs and Scientific Research Center. The plant was chopped into small pieces and dried in the shade. The dried plant was crushed to coarse powder using a hand mill and sieved. Coarse powder was successively extracted in a soxhlet apparatus with 70% ethanol for 12 h. The ethanol extracts were concentrated by evaporating under vacuum. The yield of the ethanol extract was 28.29%. The ethanol extracts were freshly dissolved in dimethylsulphoxide (DMSO) just before administration.

2.3.Cell viability assay of LNCaP cells using XTT assay

Cells were seeded at $1x10^4$ cells per well in complete culture medium containing various concentrations of ethanolic extract of *A. calamus* (0, 250, 500, 750, 1000, 1250 μg/ml and 1% DMSO vehicle control). The cells were incubated for 24 and 48 h, respectively, to determine cytotoxic and apoptotic effects. Each concentration of ethanolic extract of *A. calamus* for each incubation period was incubated in four wells to identify the most efficient dose(s) and incubation period(s). Cytotoxic activity was measured using a colorimetric assay system (XTT Cell Proliferation Kit; Biological Industries), which measures the reduction of a tetrazolium component, XTT (sodium 3′ - [1- phenyl-aminocarbonyl)-3,4-tetrazolium] bis(4-

methoxy-6-nitro)benzenesulphonic acid) into a soluble formazan product by the mitochondria of viable cells. Briefly, cells were cultured in 96-well plates containing 200 μl of complete medium. 100 μl of XTT solution was added into the wells and plates were incubated for additional 3 h. at 37 °C. The controls included native cells and medium only. Optical density (OD) of 96-well plates was measured at 450 nm in a microplate enzyme-linked immunosorbent assay (ELISA) reader (Trinity Biotech PLC, Bray CO. Wicklow, IRELAND). The percentage cytotoxicity was calculated with the formula: Percentage cytotoxicity (cell death) = [1-(absorbance of experimental wells/absorbance of control wells)] x 100%.

2.4. Apoptosis assay: Cleaved PARP (asp 214) sandwich ELISA

Cleaved PARP was assayed using the PathScan cleaved PARP (asp 214) sandwich ELISA kit (Cell Signaling Technology, Danvers, MA). Cells were seeded at $1x10^4$ cells per well in complete culture medium containing various concentrations of ethanolic extract of *A. calamus* (0, 250, 500, 750 μg/ml). The cells were incubated for 24 and 48 h, respectively, to determine apoptotic effects. Briefly, after treatment cells were washed twice with ice-cold PBS, lysed with 500 μl of ice-cold lysis buffer, scraped, and sonicated on ice. The cell lysate was then microcentrifuged for 10 min, 10.000 g at 4 °C, and the supernatant was stored at −80 °C before analysis. 100 μl of the soluble fraction was used in each well of the ELISA plate. The ELISA was performed according to the manufacturer's instructions. Absorbance was measured with a microplate reader.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR) to amplify VEGF mRNA

Total RNA was extracted from cultured LNCaP cells according to the manufacturer's instructions using the RNeasy Protect Mini Kit (Qiagen, Manheim, Germany). RNA concentration and purity was calculated after measuring absorbance at 260 nm on a UV spectrophotometer and then stored at -80°C. First-strand cDNA synthesis was performed with the QuantiTect Reverse Transcription cDNA Kit (Qiagen, Manheim, Germany). The synthesized cDNA was used as a template for PCR amplification. Quantitative PCR was performed on a LightCycler (Roche Diagnostics GmbH, Mannheim, Germany) using TaqMan technology with amplification of human glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA as a housekeeping standard. Oligonucleotide sequences of the cDNA primers were designed at Gene Research Laboratories, UK. The forward primer for VEGF was 5′-CCAGGAAAGACTGATACAGAACG-3′ and the reverse primer was 5′- GGTTTCTGGATTAAGGACTGTTC -3′. The forward primer for GAPDH was 5′-GAAGGTGAAGGTCGGAGT-3′ and the reverse primer was 5′-GAAGATGGTGATGGGATTTC-3′ The following Light Cycler conditions were used: initial denaturation at 95 °C for 10 min, followed by 50 cycles with denaturation at 95 °C for 20 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 20 s. Cycle threshold (CT) values were determined by automated threshold analysis. The primer quality (lack of primer-dimer amplification) was confirmed by melting curve analysis. Relative quantification of gene expression was performed using a

standard curve constructed with serial dilutions of control mRNA or RT-PCR amplicons. All experiments were carried out in triplicate. VEGF levels were standardized to GAPDH (ratio VEGF:GAPDH) to account for loading differences. Gene expression levels (mRNA) were reported using the median as a point estimator and the range of values.

2.6. Statistical analysis

In the cell proliferation assay, experiments were repeated three times, measurements within an experiment were done in six duplicates, and in cell cycle analysis, experiments were repeated three times. Data from three independent experiments were expressed as mean ± standard deviation (SD). Data were analyzed by a computerized probit analysis, which provided an estimate of the half maximal inhibitory concentration (IC$_{50}$). The level of significance between different groups was based on ANOVA test followed by Tukey test. All analyses were performed using SPSS 17.0 trial (IBM, USA) with P < 0.05 as the significance level.

3. Results

3.1. Effect of *A. calamus* on cell viability

The effect of *A. calamus* extract on the viability of LNCaP cells is shown in figure 1. The XTT assay showed that *A. calamus* extract decreased the viability of LNCaP cells.
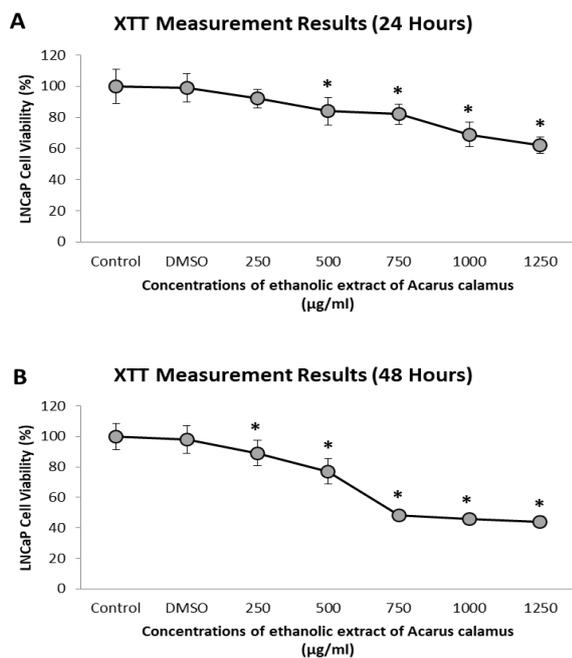
Figure 1. Effect of *A. calamus* treatment on the viability of prostate cancer cell lines LNCaP. (A) The cells were treated with various concentrations (0, 250, 500, 750, 1000, and 1250 μg/mL) of *A. calamus* for 24 h and (B) 48 h. Percentages of viable cells after treatment were determined by XTT assay. Error bars represent SD of triple samples (*P < 0.001 compared with control group). SD, standard deviation.

Acorus calamus concentrations ranging from 500 to 1250 μg/ml at 24 h and from 250 to 1250 μg/ml at 48 h showed a dose–response relationship of cell survival (24 h: control vs. 500, 750, 1000 and 1250 μg/ml; 48 h: control vs. 250, 500, 750, 1000 and 1250 μg/ml P < 0.001), while *A.*

calamus concentrations ranging from 750–1250 μg/ml showed a time-dependent decrease (750 μg/ml: 24 vs. 48h, P < 0.001; 1000 μg/ml: 24 vs. 48 h, P < 0.001; 1250 μg/ml: 24 vs. 48 h, P < 0.001). The IC$_{50}$ value was 923 μg/mL for the 48 hr treatment with extract. Based on this experiment, three concentrations (250, 500 and 750 μg/ml) lower than the IC$_{50}$ were chosen for later experiments examining the effects of *A. calamus* on LNCaP cells.

3.2. Effect of *A. calamus* on Apoptosis of LNCaP Cells

A. calamus induces apoptotic cell death in LNCaP cells. The levels of cleaved poly (ADP-ribose) polymerase (PARP), one of the best biomarkers of apoptosis, were analyzed after 24 and 48 hours of treatment with *A. calamus*. Full-length active PARP is a 116 kDa molecule, which is cleaved to fragments of 86 and 30 kDa by the action of caspase-3 and related caspases. A marked increase in cleaved PARP was observed after 24 hours of exposure to *A. calamus* at concentration of 750 μg/ml (p<0.01); 48 hours at 500 and 750 μg/ml (p<0.01, p<0.001) (Fig. 2).
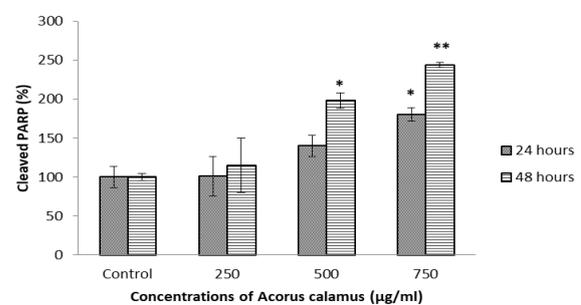
Figure 2. Effects of *A. calamus* treatment on PARP cleavage in prostate cancer cell lines LNCaP. The cells were treated with various concentrations (0, 250, 500, and 750 μg/mL) of *A. calamus* for 24 and 48 h. values represent mean ± SD (*P < 0.01; **P< 0.001 compared with control group). PARP, poly (ADP-ribose) polymerase.

3.3. Effect of *A. calamus* on VEGF mRNA expression

The dose-dependent effect of *A. calamus* extract on LNCaP cells showed a significant decrease in VEGF mRNA expression at 500 and 750 μg/ml when assessed at 48 hours post-treatment. No changes were observed after 24 hours of exposure to *A. calamus* (Fig. 3).
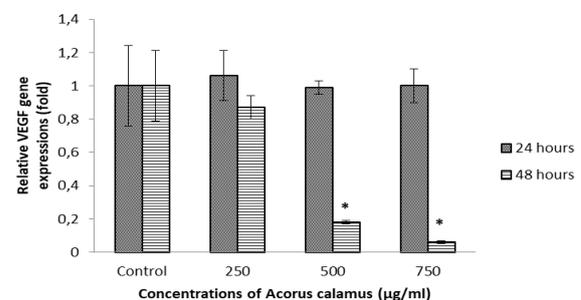
Figure 3. Effect of *A. calamus* treatment on VEGF mRNA expression in prostate cancer cell lines LNCaP. The cells were treated with various concentrations (0, 250, 500, and 750 μg/mL) of *A. calamus* for 24 and 48 h. values represent mean ± SD. (*P < 0.001 compared with control group). VEGF, vascular endothelial growth factor.

4. Discussions

Natural products have long been an important source of anticancer agents. More than 60% of drugs for cancer treatment are of natural origin, particularly derived from plants (Zhao et al., 2016). The aim of this study was to evaluate the anticancer effect of *A. calamus* extract on human prostate carcinoma LNCaP cells. We found that *A. calamus* extract inhibited the proliferation and viability of human prostate cancer LNCaP cells via induction of apoptosis. LNCaP cells showed clear apoptosis within 24 h after (750 μg/ml) and 48 h after *A. calamus* (500 and 750 μg/ml) treatment.

Apoptosis is a programmed cellular process that occurs in physiological and pathological conditions (Wu et al., 2014). Furthermore, the programmed cell death is disrupted in the cancer, which causes the malignant cells to grow too much. Induction of tumor cell apoptosis is the ultimate goal of many cancer therapies (Zhao et al., 2016). One of the cellular responses that preceded DNA repair mechanisms against DNA damage is PARP activation (Dungey et al., 2009). Anti-cancer therapies are being developed in combination with the inhibition of PARP activation and the combination of drugs that cause DNA damage (Penning et al., 2009). In the present study, we demonstrated that *A. calamus* treatment of LNCaP cells resulted in both a time and dose-dependent increase of the cleavage of PARP, which is considered a biochemical hallmark of apoptosis. However, it is not clear yet whether induction of apoptosis is mediated by pro-apoptotic factors, anti-apoptotic factors or a combined effect.

Regarding the chemical composition of *A. calamus* extract, we could hypothesize that its apoptotic effects might be dependent on the presence of lectins, volatile oils and flavonoids. Chemical analysis has demonstrated that the main constituents of *A. calamus* are monoterpenes, sesquiterpenes, phenlypropanoids, flavonoids, quinine (Kim et al., 2009). Two major active components in volatile oils of *A. calamus* are alpha-asarone and beta-asarone (Zuba and Byrska, 2012). Lectins from the rhizomes of *A. calamus* have mitogenic activity and inhibitory potential in murine cancer cell growth (Bains et al., 2005). Alfa-asarone shows radioprotective activity against lethal and sublethal doses of gamma-radiation by preventing radiation-induced production of free radicals and damage to DNA, membranes, and the hematopoietic system in animal models. Beta-asarone has anticancer effects, inhibiting survival of the human hepatoma cell line HepG2 (Kevekordes et al., 2001). Xenograft tumor models in mice showed similar results, with beta-asarone decreasing tumor size. Furthermore, survival of colorectal cancer cells *in vitro* was markedly inhibited by incubation with beta-asarone. They propose that it acts through inducing expression of lamin B1 and activating p53 (Liu et al., 2013). Beta-asarone significantly activated caspase-3, caspase-8, caspase-9, Bax, Bak and suppressed Bcl-2, Bcl-xL and survivin activity. Moreover, beta-asarone increased the expression of RECK, E-cadherin and decreased the expression of MMP-2, MMP-9, MMP-14 and N-cadherin. Beta-asarone effectively inhibits the proliferation of human gastric cancer cells, induces their apoptosis and decreased the invasive, migratory and adhesive abilities.(Wu et al., 2015). Beta-asarone can

suppress the growth of colon cancer and the induced apoptosis is possibly mediated through mitochondria/caspase pathways (Zou et al., 2012).

Angiogenesis, neovascularization from pre-existing vessels, is a key step in tumor growth and metastasis, and anti-angiogenic agents that can interfere with these essential steps of cancer development are a promising strategy for human cancer treatment (Kim et al., 2016). VEGF is the most potent known mitogen for endothelial cells, and is a specific of angiogenesis in several cancer types, including prostate cancer. Elevated VEGF levels have been observed in a variety of tumor cells (Lee et al., 2006). Prostate cancer cells produce VEGF that facilitates the growth of the tumor presumably through enhancing angiogenesis and perhaps migratory ability. VEGF promotes endothelial cell proliferation, survival, and migration (Kitigawa et al., 2005). Because prostate cancer tumors require angiogenesis for growth and metastasis, and angiogenesis is a complex multifactorial process, antiangiogenic therapy could offer a variety of avenues to arrest tumor growth, induce tumor regression, or block the ability of tumors to metastasize. Antiangiogenic therapy is a particularly attractive antitumor modality with many potential targets, considering that the regulation of tumor angiogenesis is profoundly multifactorial (Nicholson and Theodorescu, 2004).

The process of tumor angiogenesis is highly regulated by growth factors such as VEGF-A which is secreted by tumor cells and ECs and functions via paracrine and autocrine signaling pathways. After secretion VEGF-A primarily binds to its specific receptors located on ECs, triggering the process of angiogenesis (Ferrara et al., 2003). Therefore, we evaluated VEGF gene expression levels. Although we did not thoroughly investigate the mechanism, we demonstrated that VEGF expression was downregulated by treatment with *A. calamus*. Similar results indicated that *S. barbata D. Don* inhibits expression of VEGF in human colon carcinoma cells. *S. barbata D. Don* inhibits cancer growth via at least two mechanisms, inducing mitochondrion-dependent apoptosis of cancer cells and inhibiting tumor angiogenesis. Both apoptosis and angiogenesis are regulated by multi-pathways. In addition, *A. calamus* is composed of many chemical compounds including monoterpenes, sesquiterpenes, phenlypropanoids, steroides and flavonoids. It is unknown how many of these compounds contain anti-cancer activity, and we do not know which pathway(s) effected from these compounds (Wei et al., 2011).

Taken together, our study demonstrated the first evidence that *A. calamus* extract can effectively decrease proliferation, induce apoptosis and suppress VEGF-A expressions of prostate carcinoma LNCaP cells in a time- and dose-dependent manner. Therefore, although further studies are required, *A. calamus* extract may be a promising therapeutic agent for the treatment of prostate tumors.

Conflicts of interest

There is no conflict of interest in any form between the authors.

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