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Investigation of Cytotoxic Effect of *Salvia pilifera* Extracts and Synthetic Chlorogenic and Caffeic Acids on DU145 Prostate Cancer Cells Line

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ABSTRACT

Salvia species have been used in the treatment of many diseases due to their medical effects, and the effects of these species on prostate cancer cells should be investigated in more detail. In this study, we aimed to determine anti-carcinogenic activities of dichloromethane (DCM) and methanol (MeOH) extracts of *Salvia pilifera* and the synthetic chloregenic (CGA) and caffeic acids (CA) on DU-145 prostate cancer cells. The cytotoxicity of extracts and synthetic compounds on cell viability of DU-145 was measured by using MTT method. Induction of apoptosis was tested by using Annexin V and 7ADD staining. DNA fragmentation was evaluated in cells. Also, transcripton levels of Bax, Caspase 3, Caspase 9 and Bcl-2 and Bcl-xL genes were determined. Lastly, the phenolic compounds in MeOH extract were determined by HPLC. In MTT test, extracts, CGA and CA were found to be diminished proliferation of DU145 cells. However, in apoptosis assay, no apoptotic activity for extract and synthetic compounds was observed. In DNA fragmentation test, while no significant difference in extracts group was observed as compared to controls, fragmentation as swab in synthetic compound groups was observed. Small changes were observed in transcription levels of apoptotic and antipoptotic genes. A total of 11 phenolic acids were determined including fumaric acid, gallic acid, gallocatechin, catechin, oleorufein, 4-hydroxybenzoic acid, caffeic acid, syringic acid, ellagic acid, 3-hydroxy cinnamic acid and protocatechuic acid. Results of the present study suggest that *S. pilifera* extracts and synthetic CGA and CA might have cytotoxic effects on DU145 cell at certain concentration (≥50 µg ml^{-1} for DCM; ≥ 100 µg ml^{-1} for MeOH; ≥ 1 µg ml^{-1} for CGA and CA) yet that these effects may be manifested through another pathway but apoptosis.

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Prostat Kanser Hücre Hattı DU145 Üzerine *Salvia Pilifera* Ekstraktları ile Sentetik Klorojenik ve Kafeik Asidin Sitotoksik ve Apoptotik Etkilerinin Incelenmesi

ÖZET

Salvia türleri tıbbi etkilerinden dolayı pek çok hastalığın tedavisinde kullanılmaktadır, Ancak bu türlerin prostat kanseri üzerine etkilerini gösteren çalışmaların sayısı sınırldır. Bu çalışmada, *S. pilifera* diklorometan (DCM) ve metanol (MeOH) ekstraktları ile sentetik klorojenik (CGA) ve kafeik asitin (CA) DU145 prostat kanser hücreleri üzerine antikanser aktivitelerinin belirlenmesi amaçlanmıştır. DU145 hücrelerinin canlılığı MTT boyama testi ile belirlenmiştir. Apoptozun indüklenmesi Annexine V ve 7ADD boyama kiti kullanılarak belirlenmiştir. Özüt uygulaması sonrasında hücrelerin DNA'ları izole edilerek

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Araştırma Makalesi

fragmentasyona bakılmıştır. Son olarak, BAX, Kaspaz3 ve 9, BcL2 ve BcL-xL gen ekspresyon seviyeleri ölçülmüştür. Bununla birlikte, MeOH ektraktındaki olası fenolik bileşikler HPLC ile belirlenmiştir. MTT testinde, ekstrakt ve sentetik fenoliklerin DU145 hücrelerinin canlılığını azalttığı tespit edilmiştir. Ancak apoptoz indükleme testinde ekstrakt ve fenoliklerin herhangi bir aktivite sergilemedikleri belirlenmiştir. DNA fragmentasyon testinde, kontrolle kıyaslandığında ekstrakt uygulanan gruplarda bir farklılık görülmezken, CGA ve CA uygulama gruplarında sürüntü şeklinde parçalanma gözlenmiştir. Ayrıca, apoptotik ve antiapoptotik gen ekspresyonlarında zayıf değişimler gözlenmekle birlikte bu değişimler istatistiksel olarak önemli bulunmamıştır. Ayrıca, *S. pilifera*'da fumarik asit, gallik asit, gallokateşin, kateşin, oleorufein, 4-hidroksibenzoik asit, kafeik asit, sirinjik asit, ellajik asit, 3-hidroksi sinnamik asit ve protokateşik asit belirlenmiştir. Sonuç olarak, *S. pilifera* DCM ve MeOH özütleri ve kullanılan sentetik fenolik asitlerin prostat kanser hücreleri üzerine belirli dozlarda (DCM için ≥50 µg ml^{-1}; MeOH için ≥ 100 µg ml^{-1}; CGA and CA için ≥ 1 µg ml^{-1}) sitotoksit etki gösterdikleri, ancak bu etkinin apoptoz yolağıyla ilişkili olmadığı belirlenmiştir.

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INTRODUCTION

Prostate cancer is one of the leading causes of death among male population. Although several types of treatment strategies have been utilized to fight against this cancer type (Morote et al., 2016), the adverse effects of current strategies such as chemotherapy and radiotherapy is very high to be ignored. Due the adverse effects of synthetic chemotherapeutics, developing novel natural anti-cancer agents with low toxic effects is very important (Unnati et al., 2013). In this regard, the number of studies on plant-derived agents that may have the potential anticancer effects has increased considerably (Ren et al., 2016; Wu et al., 2017). The members of Lamiaceae family of plants have been used widely in numerous anticancer studies (Russo et al., 2013; Sghaier et al., 2016). Plants belonging to the Lamiaceae family have long been used in many countries as tea and spices. One of the most popular genus of this family is Salvia and estimated to have nearly 900 species in the world (Walker et al., 2004). This genus spread to Turkish flora with 94 taxa and 45 of them is endemic (Davis, 1982). The seconder metabolites of plant that belongs to this genus are highly divergent (Lu and Yeap, 2002). Accumulating body of evidence suggest that Salvia species has several biological activities including antioxidant (Yumrutas et al., 2012), antimicrobial (Bahadori et al., 2015), anti-inflammatory (Jung et al., 2009), analgesic (Amabeoku et al., 2001), and anticancer (Russo et al., 2016) influences. Additionally, species of this genus was reported to be used in many anticancer studies. In a study, Farimani et al. (2015) was reported that triterpenoids of *S. urmiensis* shows anticancer activities on HeLa and HepG2 cancer cells and induced apoptosis by regulating Bcl2 family of proteins. In a different study, methanol extracts of *S. chinensis*, which is rich in polyphenols, was reported to induce apoptosis and cell cycle arrest at G0/G1 in pancreatic cancer cells, showing strong anticancer activities (Zhao et al., 2015). It has been reported that the Salvia species growing in Turkey have various biological activities including antioxidant (Koşar et al., 2011), antimicrobial (Tepe et al., 2005) and acetylcholinesterase inhibitory (Tel et al., 2010) activities. But, the information about anticancer activities of Salvia species is very limited. *S. pilifera* is an endemic species and was reported to have antioxidant and antimicrobial activities (Kelen and Tepe, 2008). Yet, its anticancer activity remains elusive. Accordingly, in the present study, we aimed to evaluate anticancer activities of semi polar and polar extracts of *S. pilifera* on a prostate cancer cell line, DU-145.

MATERIAL and METHODS

Preparation of extracts

For the preparation of Dichloromethan (DCM) and Methanol (MeOH) standard Soxhlet extraction method was followed (Sokmen et al., 1999). Briefly, plant materials (40 g) were air-dried and then the samples was extracted in a Soxhlet apparatus with DCM (400 ml) and MeOH (400 ml) at 40 and 50 ºC for 6 h, respectively. Both extracts were then concentrated by using a rotary vacuum evaporator at 45 ºC. Finaly, the extracts were kept in the dark at +4 ºC until further experiments.

MTT cell viability assay

For the determination of cell viability of DU-145 cells, MTT (3- [4,5- dimethylthiazol- 2- yl]- 2,5- diphenyl- tetrazolium bromide) cell viability assay was used. DU-145 were seeded to 96-well plates with a $1x10^6$ ml^{-1} and incubated for a period of 24 hours with 25, 50, 100, 150 µg ml^{-1} doses of extracts and 0.2, 1, 5 and 25 µg ml^{-1} doses of phenolic acids. After 24 hours, cells were washed with PBS and treated with 1 mg ml^{-1} MTT (Sigma) dissolved in growth medium and incubated at 37°C for 45 minutes. Subsequently, MTT solution was removed and dimethyl sulfoxide (DMSO) (Sigma-Aldrich, MO, USA) was used to dissolve absorbed dye. Immediately, plates were read at 570 nm using an EZ read 400 microplate reader (Biochrom, Cambridge, UK). The experiment was repeated four times.

Determination of Apoptosis via Annexin V/7ADD staining

For the apoptosis analysis by Annexin V/7ADD staining, cells were seeded at a $1x10^6$ ml^{-1} density to 6-well plates and active doses of extracts and phenolic acids were applied for a period of 24 hours. Subsequently, cells were trypsinized and subjected to Annexin V/7ADD staining and analyzed at flow cytometry (Beckman Coulter). This experiment was repeated two times.

DNA fragmentation analysis

DNA fragmentation analysis was performed according to Yang et al. (2000). Prostate cancer cells were seeded at a $3x10^6$ ml^{-1} density and incubated with the various dosed of DCM and MeOH extracts for a period of 24 hours. Cells were harvested after 24 hours and subjected to DNA isolation by standard phenol-chloroform method. And then the isolated DNAs were run on a 2.0% of agarose for electrophoresis (Thermo Scientific, Germany) and visualized under UV light imaging system (Vilber Lourmat, France)

Gene transcription analysis by qPCR

Gene transcription levels of apoptotic and anti-apoptotic genes were determined by using real-time PCR method. Briefly, cells were incubated with the appropriate doses for period of 24 hours and subjected to total RNA isolation. RNA samples were further converted to cDNA by using first strand cDNA synthesis kit. All the transcription reactions were performed by using thermos maxima syber green kit in Rotor Gene 6000 Real-Time PCR. This experiment was repeated three times. The primer pairs used are given in Table 1.

Determination of phenolic acid contents of extracts

For the determination of phenolic acid content of extracts high-performance liquid chromatography (HPLC) method was used. For the analysis, ChemStation software, G1322A model degasser, G1311 model quaternary pump, G1329 model auto-sampler device and G1321 model fluorescence detector were used. The separation was performed using Zorbax Eclipse XDB-C18 model columns (150 mm, 4.6 mm and 5 m particle width) (Shimadzu, Waldbronn, Germany).

Table 1. Primer pairs used in the amplification of apoptotic and anti-apoptotic genes.

Genes		Primer sequences
Bcl-xL	Forward	5-CCCAGAAAGGATACAGCTGG-3
	Reverse	5-GCGATCCGACTCAC-CAATAC3
Bcl-2	Forward	5-GAACTGGGGGAGGATTGTGG-3
	Reverse	5-CCGGTTCAGGTACTCAGTCA-3
Caspase3	Forward	5-AGAGGGGATCGTTGTAGAAG-3
	Reverse	5-GTTGCCACCTTTCGGTTAAC-3
Caspase9	Forward	5-TGTTCAGGCCCCATATGATCG-3
	Reverse	5-GGAAAGCTTTGGGGTGCAAG-3
Bax	Forward	5-GATGATTGCCGCCGTGGAC-3
	Reverse	5-GGGTGAGGAGGCTTGAGGAG-3
GAPDH	Forward	5-GAAGGTGAAGGTCG-GATGC-3
	Reverse	5-GAAGATGGTGATGGGATTTC-3

Statistical analysis

For the statistical evaluation of data GraphPad Prisim and SPSS package softwares were used. Gene transcription levels of genes were calculated using the formula ; $2^{-\Delta Ct}$ (ΔCt = CT $_{Target\ gene}$ − CT $_{Reference\ Gene}$). Normalization of the gene transcription data was achieved using GAPDH as a reference gene. For all statistical analysis, p values were two-tailed and $p<0.05$ accepted as statistically significant.

RESULT and DISCUSSION

For the investigation of anticancer activities anti-proliferative, apoptotic and related gene transcriptions were assessed after extract treatments. Additionally, phenolic acid constituents of each extracts were determined. Anticancer activities of CGA and CA were also determined.

Anti-proliferative activities of *S. pilifera* extracts, CGA and CA

To assess anti-proliferative activities of *S. pilifera* extracts, CGA and CA MTT cell viability assay was

performed. For the determination of cell viability of DU-145 prostate cancer cells various concentrations of extracts, CGA and CA were used. For the DCM and MeOH extracts of *S. pilifera* 25, 50, 100 and 150 µg ml^{-1} doses were used. For the CGA and CA 0.2, 1, 5 and 25 µg ml-1 application doses were used. As a result, DCM extract of *S. pilifera* was found to inhibit proliferation of DU-145 prostate cancer cells in a dose-dependent manner (Figure 1). In the statistical analysis, changes in 100 and 150 µg ml^{-1} doses were found to be statistically significant ($p<0.01$). Similar to DCM extract, MeOH extract also showed anti-proliferative activity at 100 µg ml^{-1} ($p<0.05$) and 150 µg ml^{-1} ($p<0.01$) doses. As a result, CGA showed highest activity at 25 µg ml^{-1} doses ($p<0.01$), while 1 and 5 µg ml^{-1} ($p<0.05$) doses was showed nearly same activity. Likewise, all of doses of CA showed highest anti-proliferative activity on prostate cancer cells ($p<0.01$). Accordingly, doses were chosen to be 150 µg ml^{-1} for DCM extract and MeOH extract and 25 µg ml^{-1} for CGA and CA. Accumulating mass of indication suggest that some species of salvia inhibits proliferation of cancer cells (Russo et al., 2013; Russo et al., 2016). In addition, CA was reported to inhibit proliferation of HT-1080 cells (a human fibrosarcoma cell line) and altered mitochondrial membrane hemostasis (Prasad et al., 2011). Consistent with the previous findings, in our study, we also revealed that *S. pilifera* and CA show significant anti-proliferative activities.

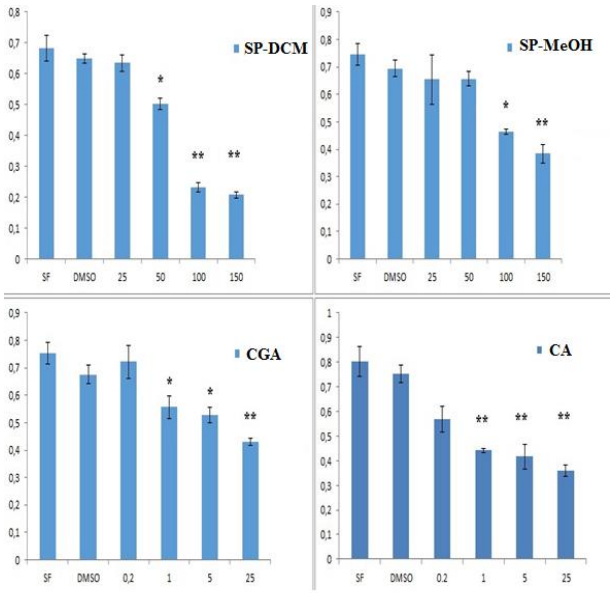

Figure 1. Antiproliferative effects of DCM and MeOH of *S. pilifera,* CGA and CA on DU145 prostate cancer cells. SP-DCM: *S. pilifera* DCM extract, SP-MeOH: *S. pilifera* MeOH extract

Induction of apoptosis in DU-145 cells after extract and synthetic phenolic compounds treatments

Apoptosis is accepted as a natural defense mechanism against cancers. It is evident that plants and their phytochemicals induces apoptosis and inhibits proliferation of cancer cells, showing significant anticancer activities (Mei et al., 2016, Weidner et al., 2015). To reveal which type of cell death induced after *S. pilifera* extract treatments, we used Annexin V/7ADD double staining assay. As a result, apoptotic programmed cell death was found to be not induce after DCM and MeOH extracts treatments (Figure 2). Similar to *S. pilifera* plant extracts, phenolic acids of CGA and CA did not induced apoptosis in DU-145 cells. However, significant necrotic effect was observed in CA treated cells. CGA In previous studies, various species of Salvia was reported to induce apoptosis in different types of cancer cells. In particular, ethanol extract of *S. chinensis*, which is rich in phenolic acids, was reported to be induced apoptosis in pancreatic cancer cells (Zhao et al. 2015).

Determination of DNA fragmentation

In addition, Russo et al. (2016) reported that essential oils of *S.aurea, S. judaica* and *S. viscosa* induce apoptosis by activating caspases. In addition to, CGA was reported to be interfered with proliferation of A549 lung cancer cells and played a chemo-preventive role by the suppression of NF-κB, AP-1, and MAPK activation (Feng et al., 2005). Moreover, Granado-serrano et al. (2007) postulated that CGA treatments in human hepatocellular carcinoma cell line (HepG2) are not associated with the BCL-2 and BAX transcription s, thus in turn, not associated with the apoptosis. Consequently, different species of Salvia genus seems to have different biological activities. Therefore, it can be concluded that DCM extract, MeOH extract, CGA and CA have no apoptotic activity in DU145 prostate cancer cells.

To confirm the results of the Annexin V/7ADD double staining assay, we also performed DNA fragmentation assay. DNA fragmentation is one of the indicators of cellular apoptosis (Kerr and Winterfold, 1994). As a result of the DNA fragmentation assay, both extracts and phenolic acids (CGA and CA) were found to be not induced fragmentation of DNA in DU-145 cells. In consistent with the results of Annexin V/7ADD double staining assay, DCM extract and MeOH extract of *S. pilifera* was found to be not successful in triggering apoptotic cell death. Also, in phenolic acid treated cells, smear-like banding pattern observed. The reason behind this observation can be the uncontrolled DNA fragmentation resulted from necrotic cell death as we observed in flow cytometric analysis (Figure 3) (Kerr and Winterfold, 1994).

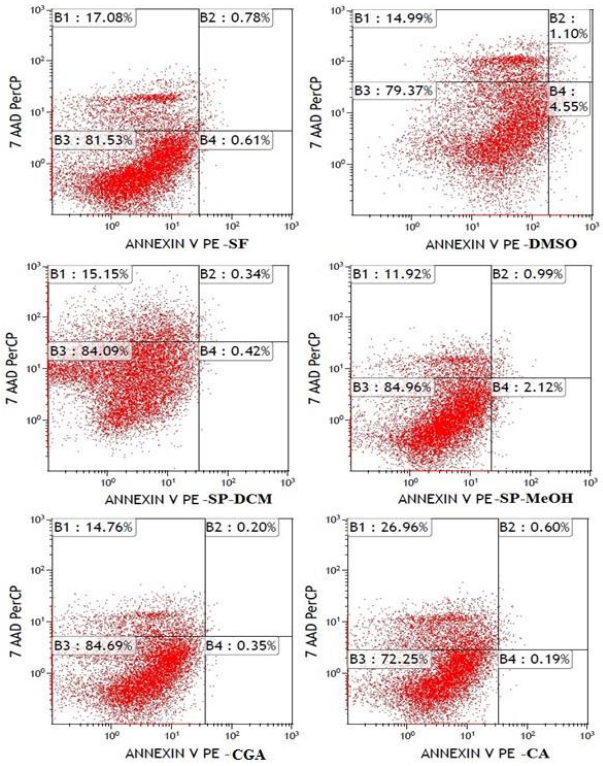

Figure 2. Apoptosis induction effects of DCM and MeOH extracts of *S. pilifera*, CGA and CA on DU145 prostate cancer cells. SPM: *S. pilifera* MeOH extract, SPD: *S. pilifera* DCM extract, B1: Necrotic cells, B2: Late apoptosic cells, B3: uneffected cancer cells, B4: Early apoptotic cells

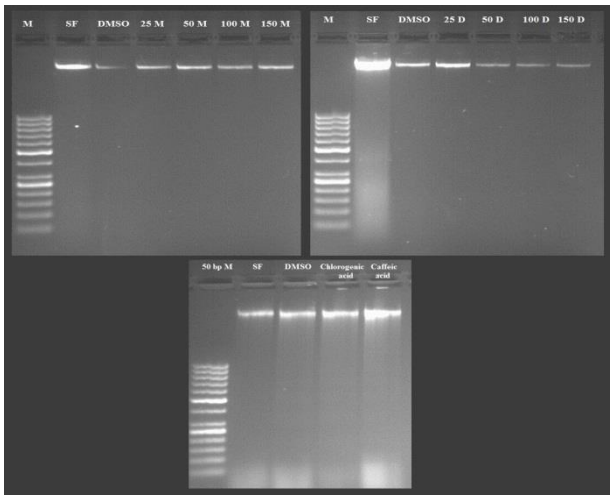

Figure 3. DNA fragmentations effects of DU145 cells after treatment of DCM and MeOH extracts of *S. pilifera,* CGA and CA. M: marker, 25D-150D: DCM extract doses, 25M-150M: MeOH extract doses.

Transcription levels of apoptotic and anti-apoptotic genes

In living cells, apoptosis, as it is also called programmed cell death, is a programmed mechanism which is tightly coordinated by the transcription of several pro-apoptotic (BAX, BID, and BAK) and anti-apoptotic (such as BCl-2 and BCL-xL) genes. In normal circumstances, the levels of pro-apoptotic and anti-apoptotic proteins are kept in balance (Johnstone et al. 2002). Yet, under abnormal circumstances such as irreversible DNA damage, the apoptosis mechanism is triggered by transcription pro-apoptotic proteins (Johnstone et al., 2002). In our study, to investigate whether apoptosis is induced at molecular level, transcription levels of apoptotic (Caspase 3, 9 and BAX) and anti-apoptotic (BCl-2 and BCL-xL) were determined after extract and phenolic acid treatments as presented Figure 4.

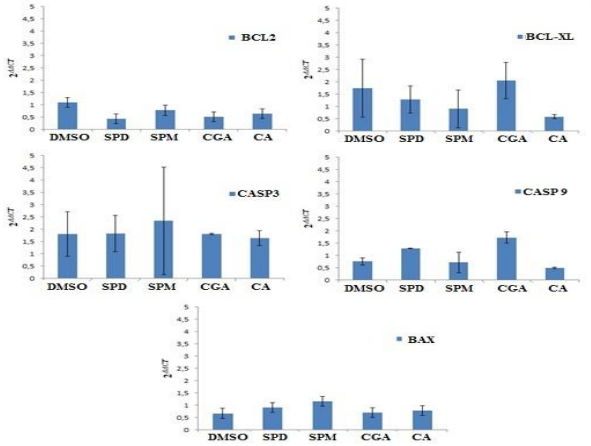

Figure 4. Effects of DCM and MeOH extracts, CGA and CA on transcription of apoptotic and antiapoptotic genes. SPM: *S. pilifera* MeOH extract, SPD: *S. pilifera* DCM extract

Particularly, no significant transcription change was observed after extract and phenolic acid treatments. In addition, transcription levels of Caspase 9 and BCL2 were found to be slightly increased in CGA treated cells, yet these changes were statistically insignificant ($p>0.05$). Ali et al (2017) reported that CGA exhibited anti apoptotic effect due to decrease levels of the apoptotic markers including Bax, BCL-2 and Casp 3, 9. In previous studies, salvia species were reported to effect transcription levels of apoptotic and anti-apoptotic genes. In particular, Farimani et al (2015) reported that transcription levels of BCL2 and BCL2 family of genes were reduced and BAX increased in HeLa and HepG2 cancer cells after treatment with triterpenoids of *S. urmiensis*. Suggesting that triterpenoids of *S. urmiensis* induces apoptosis by altering transcription levels of pro-apoptotic and anti-apoptotic genes, showing significant anti-cancer activity. Moreover, Russo et al (2016) reported that apoptosis was induced in melanoma cells by the increased caspase 3 transcription levels after treatment with the essential oils of Salvia species. In addition to, in prostate cancer cells tanshinone 1 which is isolated from *S. miltiorrhiza*, was reported to induce

apoptosis by reducing BCL2, and increasing BAX gene transcription (Gong et al., 2011). Taken together, the results of the current study suggest that *S. pilifera* plant extracts, CGA and CA phenolic acids have no apoptotic activity.

Phenolic acid contents of extracts

A growing body of evidence suggest that salvia species contains a significant amount and variety of phytochemicals. Additionally, phytochemicals derived from this species was reported to be significantly associated with the increased apoptotic activity (Farimani et al., 2015; Giacomelli et al., 2013). To determine phenolic acids present in MeOH extract of *S. pilifera* high-performance liquid chromatography (HPLC) method was used. As a result, a total of 11 phenolic acids were determined including fumaric acid, gallic acid, gallocatechin, catechin, oleorufein, 4-hydroxybenzoic acid, CA acid, syringic acid, ellagic acid, 3-hydroxy cinnamic acid and protocatechuic acid as presented in Table 2.

Table 2. Phenolic acids determined in MeOH extract of *S. pilifera* by HPLC analysis.

Phenolics	Concentration (mg kg^{-1})
Fumaric acid	116,094
Gallic acid	0,061
Gallocatechin	6,967
Catechin	0,732
Oleorufein	94,113
4-hydroxybenzoic acid	0,211
CA acid	3,815
Syringic acid	6,132
Ellagic acid	3,088
3-hydroxy cinnamic acid	0,290
Protocatechuic acid ethyl ester	1,220

Moreover, fumaric was found to be most abundant compound. Also, oleorufein was the second most abundant compound in extracts. In addition, 1,8-cineol, borneol and camphor, alpha and beta pinene, caryophyllene oxide and thymol volatile oils were determined. Consistent with these findings, Orhan et al. (2012) in their study determined p-hydroxybenzoic acid, valinic acid, CA acid, CGA acid, ferulic acid, rosmarinic acid and t-cinnamic acid in MeOH extract of *S. pilifera* by HPLC analysis.

CONCLUSIONS

In conclusion, results of the present work shows that DCM and MeOH extracts of *S. pilifera* possess no anticancer activity on prostate cancer cells. Additionally, CGA and CA phenolic acids were shown to have no pro-apoptotic activity on prostate cancer cells. Although both extracts and phenolic acids strongly inhibit the proliferation of prostate cancer cells, the mechanisms of cell death are not much related to apoptosis. Considering a whole plant extract is composed of a variety of phytochemicals, determination of individual anticancer activities of these chemicals is very important to understand their potential therapeutic use in cancer. In the future studies, it is highly recommended that isolation and characterization of active compound present in extracts is of great interest.

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