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ORIGINAL ARTICLE

Androgen Receptor (NR3C4) Regulator Potential of *Ceratonia siliqua* Extract and Its Signaling Pathways

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ABSTRACT

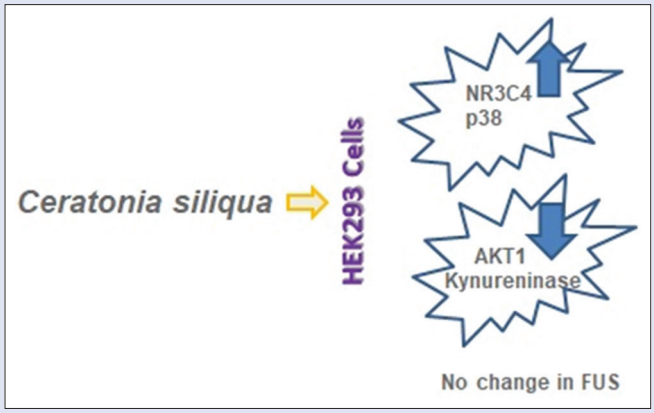
Aim/Background: Androgen receptor (AR/NR3C4) regulates growth, development, reproduction, metabolism, and homeostasis. Since it has multifunctional properties, disrupting of the functions can cause many diseases such as cancer and neurodegenerative diseases. Therefore, AR modulators and blockers are very important therapy of androgen-dependent diseases. Furthermore, mechanisms and signaling pathways of AR on the diseases are unclear. *Ceratonia siliqua* was shown to have preventative effects against digestive system disorders, diabetes mellitus, asthma-bronchitis, and oxidative stress in various studies. **Materials and Methods:** In the present study, its AR regulator potential was investigated in AR-deficient HEK293 cells. **Results:** While *C. siliqua* extract increased >2 folds of NR3C4 gene expression in the cells, it induced a decrease in AKT1 and kynureninase genes expression levels. On the other hand, it increased p38 gene expression, but it did not change FUS gene expression. These results support a regulating role on the receptor and also that the compounds specifically affect NR3C4 signaling pathways. **Conclusion:** Determination of the molecules and their various combinations with each other can contribute to discover new therapeutic agents for diseases dependent on AR signaling pathways.

Key words: AKT1, androgen receptor (NR3C4), *Ceratonia siliqua*, kynureninase

SUMMARY

- This is the first study showing the effects of *Ceratonia siliqua* extract and its signaling pathways.

Abbreviations used: AF-1: Activation factor 1; AR: Androgen receptor; DBD: DNA-binding domain; HPRT: Hypoxanthine-guanine-phosphorib osyltransferase; LC_{50}: Lethal concentration killing 50% of cells; LBD: Ligand-binding domain; NTD: N-terminal region; ATCC: The American Type Culture Collection; SPSS: Statistical Package for the Social Sciences.



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INTRODUCTION

Androgen receptor (AR/NR3C4) is a member of the nuclear receptor superfamily consisting of 48 members and regulates growth, development, reproduction, metabolism, and homeostasis.[1-4] Since it has multifunctional properties, disrupting of the functions, can cause many diseases such as cancer and neurodegenerative diseases. Therefore, AR modulators and blockers are very important therapy of androgen-dependent diseases. On the other hand, AR receptor can be activated by cytokines, growth regulators, or nonsteroidal molecules in case of absence or low concentrations of androgen.[5-7] Furthermore, mechanisms and signaling pathways of AR on the diseases are unclear.

NR3C4 receptor has three functional domains. These are the N-terminal region (NTD), DNA-binding domain (DBD) and terminal region with ligand-binding domain (LBD). N-terminal region (NTD), activation factor 1 (AF-1), containing a transactivation domain independently of its ligand and is encoded by the 1st exon. DBD containing two zinc finger motifs through four cysteines is encoded by the 2nd and 3rd exons on the forward 72 amino acids and forms 10% of the entire receptor. Terminal region with LBD is encoded by 4–8 exons.[8-11] *AR/NR3C4* gene encoding the AR are located on the X chromosome; it is located in the para-centromeric region on the long arm between q11 and q13 with a length of about 90 kb with 8 exons.[5] AR gene sequences contain palindromic, repetitive sequences. These repetitions are in the form of CAG strings. Glutamine (Q), which is found in the protein structure, is synthesized by being encoded by gland-repetitive codons (CAG). The length of CAG sequences shows differences between individuals.[12-14]

Ceratonia siliqua is an evergreen, broad-leaved plant belonging to the family *Leguminosae.* It has been argued that has preventative effects against digestive system disorders, diabetes mellitus, asthma-bronchitis, and oxidative stress.[15-18] Furthermore, it was used for fertility in Anatolia as traditional medicine. This plant includes many substances

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such as polyphenolic compounds, arachidonic acid, lignin, fats, proteins, carbohydrates, aspartic acid, glutamic acid, linolenic acid, linoleic acid, Vitamin E, beta-sitosterol, calcium, potassium, silica, iron, magnesium, and phosphorus (Mokhtari *et al*. 2012). Therefore, the plant including rich ingredients has a high potential to discover new agents and their combinations against various diseases. In this study, effects of the extract on AR gene expression and its signaling pathways were investigated in AR-deficient HEK293 cells.

MATERIALS AND METHODS

Preparation of *Ceratonia siliqua* extract

Fruits of *C. siliqua* were used for the extract preparation. Fruits were air-dried at room temperature. Dried fruits were extracted by using methanol in an Soxhlet extractor. Methanol extracts were evaporated using rotavapor. Extracts were dissolved in water using an ultrasonic bath and filtered. The filtrate was treated with 3% H_2SO_4 to a final pH of 3–4, followed by a chloroform extraction. Chloroform extracts were dried on anhydrous Na_2SO_4, filtered and concentrated under vacuum.

Mammalian cell culture

HEK293 cells were used in this study. It is a specific cell line formed by culturing human embryonic kidney cells under laboratory conditions. The American Type Culture Collection (ATCC) code number ATCC® CRL-1573TM. These cells also have the NR3C4 receptor, which is the receptor for the androgen hormone, due to their similarity to the adrenal cortex and used as cell lines in studies such as the AR.[19] Cells were grown in Dulbecco's Modified Eagle Medium containing penicillin/streptomycin, L-glutamine, and 10% fetal bovine serum.

Cell viability assay

3-(4,5-dimethylthiazole-2yl)-2,5-diphenyl tetrazolium bromide (MTT) cytotoxicity test was performed to determine the appropriate dose range of the prepared *C. siliqua* extract for experiments and HEK293 cells with human embryonic kidney cells were used for the test.

HEK293 cells were counted and adjusted to 100,000 cells per ml. A volume of 10 µl of extract dilutions; with six different concentrations as 500, 50, 10, 5, 1 µg/ml and 90 µl of cells were added to the wells. Cells were incubated for 2 h in a humidified environment at 37°C in a 5% CO_2 incubator for 4 h after the addition of 10 µl MTT (5 mg/ml) to each well. After incubation, 80 µl of the supernatant in the wells was withdrawn and 100 µl of 50% solution of sodium dodecyl sulfate (SDS, pH 5.5) dissolved in isopropyl alcohol was added to the wells. The prepared SDS mixture destroys the formazan crystals formed by MTT. The resultant color was measured at 570 nm with respect to the reference wavelength of 630 nm using the ELISA spectrometer.[20,21] As controls, cells incubated with the medium were used. The cytotoxicity index of the new extracts was compared to the control and the cytotoxicity index was calculated. In addition, the efficacy of the extracts was compared by calculating the lethal concentration killing 50% of cells from the dose-response curve.

RNA isolation

RNA isolation experiments were carried out after the incubation of *C. siliqua* extracts with HEK293 cells at the appropriate concentration determined according to the cytotoxicity test. For 107 cells, 1 ml of tri-reagent was placed, pipetted, and allowed to stand at room temperature for 5 min. 200 (mu) l of chloroform was added, vortexed for 15 s and allowed to stand at room temperature for 3 min. It was centrifuged at +4°C for 15 min and the upper phase was replaced with a new one. A volume of 500 µl of propanol was added and vortexed and allowed to stand at room temperature for 10 min. Centrifuged for 10 min at +4°C and supernatant was discarded and 750 µl 75% ethanol was added. Centrifugation at 5 min at +4°C was performed and the supernatant was again discarded and incubated for 10 min on ice. The alcohol was dissolved by adding 30 µL DEPC H_2O at the end and stored at −80°C. cDNA synthesis was carried out using SensiFAST cDNA Synthesis Kit according to their manufacturer instructions.

Real time polymerase chain reaction

Gene expression levels of NR3C4, AKT1, FUS, p38, and kynureninase were measured by using Roche LightCyclere FastStart DNA Master SYBR Green I kit according to the manufacturer instructions. For NR3C4 gene, forward primer was 5 CCTGGCTTCCGCAACTTACAC3C and reverse primer was 5 GGACTTGTGCATGCGGTACTCA3GA CTTGTGCATGCGGTACTCAs 5 gene, forwar5GACCTCAGCCCAC CCTTCAA3T and reverse primer was 5aGCTGTCCACAC ACTCCATGCT3C. For FUS gene, forward primer was 5aAAACAAGAAAACGGGACAGC3A and reverse primer was 5rGGCGAGTAGCAAATGAGACC3n. For p38 gene, forward primer was 5asmerdGTAGCAAATGAGACC3no and reverse primer was 5rGGCGAGTAGCAAATGAGACC3n. Furthermore, the forward primer 5as 5rGGCGAGTAGCAAATGAGAC and reverse primer 5as 5rGGCGAGTAGCAAATGAGACC3n. For p38 gene, forkynureninase gene expression levels. Hypoxanthine-guanine-phosphoribosyltransfer ase gene assay (Roche Life Science) was used for normalization.

Statistical analysis

Statistical Product and Service Solutions (SPSS) program was used for statistical analysis of the results. Statistically significant differences were analyzed using the Student's *t*-test. Analyzes were statistically significant with statistical significance of $P < 0.05$.

RESULTS AND DISCUSSION

First, cytotoxicity of *C. siliqua* extract against HEK-293 cells was measured using the MTT colorimetric method. According to cytotoxicity results, it was found that it is cytotoxic against the cells at 500 µg/ml concentration [Figure 1]. Therefore, to maintain the viability of the cells, the gene expression experiments were made at 10 µg/ml concentration.

Before and after *C. siliqua* extract incubation in HEK293 cells, NR3C4, AKT1, FUS, P38, kynureninase genes expression levels were measured. *C. siliqua* extract increased >2 folds of NR3C4 gene expression in the cells [Figure 2]. Although NR3C4 expression levels of the cells are insufficient,

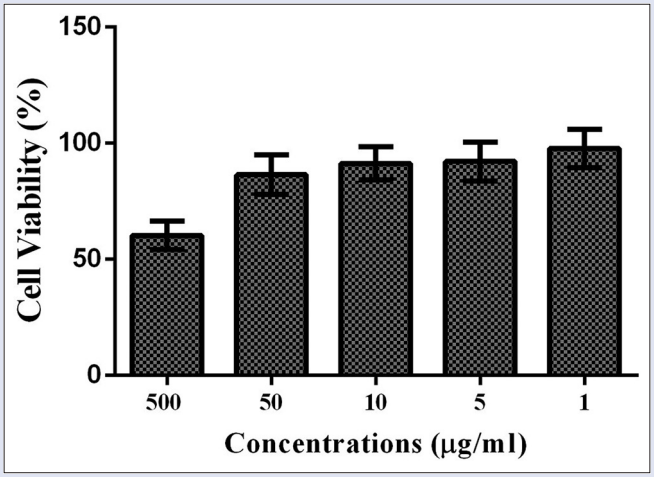

Figure 1: Cytotoxicity of *Ceratonia siliqua* extract on HEK-293 cells

the increasing effect of the extract can shed a light understanding of new signaling pathways in NR3C4 receptor gene regulation.

On one hand, the extract induced a decrease in AKT1 gene expression levels [Figure 3]. According to the results, the extract suppresses AKT1 gene expression as independent from AR. Since the extract contains various bioactive molecules, isolation of the molecules from the extract and their different combinations with each other can help to discover a new suppressor agent, specifically affecting AKT1 gene expression. AR antagonists used in prostate cancer therapy can lead to negative effects on other functions of the receptor and cause androgen-independent prostate cancer transition. Therefore, suppression of the signaling nodes advancing cancer progression as independent from androgen receptor is very important to prevent adverse effects of the antagonists.

On the other hand, *C. siliqua* extract incubation did not change FUS gene expression levels in HEK293 cells [Figure 4]. It was shown that FUS is co-activator of AR in LNCaP human prostate cancer cells.[22] Since it is target for AR, its neurodegenerative effects were also studied in a different study. However, FUS expression did not change in the presence of the poly-Q expanded AR.[23] According to the studies, FUS is not the main regulator of AR, but it can contribute to the signaling pathway of androgen-dependent cell proliferation in prostate cancer cells. Although the extract increased AR gene expression, it did not affect FUS gene expression. The results showed that the ingredients of the extract interacted with different signaling nodes to activate AR gene expression.

According to our results, the extract significantly increased p38 genes expression levels in HEK293 cells [Figure 5]. Although the AR is mainly activated by androgens, it has been found that AR can also be activated by cytokines, growth factors either nonsteroidal molecules in very low concentrations or the absence of androgen.[5-7] The p38/MAPK signaling pathway activated by interleukin-6, insulin-like growth factor type 1, and epidermal growth factor induces AR activation.[24-26] We found that the extract increased p38 gene expression. Furthermore, this effect may contribute to increase >2 folds of NR3C4 gene expression in HEK293 cells.

Tryptophan catabolism plays a role in many chronic inflammatory pathologies such as cancer, chronic infection, allergies, neurological disorders, and autoimmunity.[27,28] In kynurenine pathway, kynureninase leads to 3-hydroxyanthranillic acid and quinolinic acid being a N-methyl-D-aspartate agonist productions. Therefore, it can mediate neuronal atrophy dependent on glutamate toxicity. In our study, *C. siliqua* extract also decreased kynureninase gene expression in HEK293 cells [Figure 6].

Different extracts and antioxidants have been reported affecting gene regulation of living organisms and ameliorates damages caused by oxidative stress.[29-34] *C. siliqua* plant has been used for many years in Anatolia by traditional methods in the treatment of many diseases. However, its effects on NR3C4 gene regulation are unknown. The initial screening study showed that the extract increased >2 folds of NR3C4

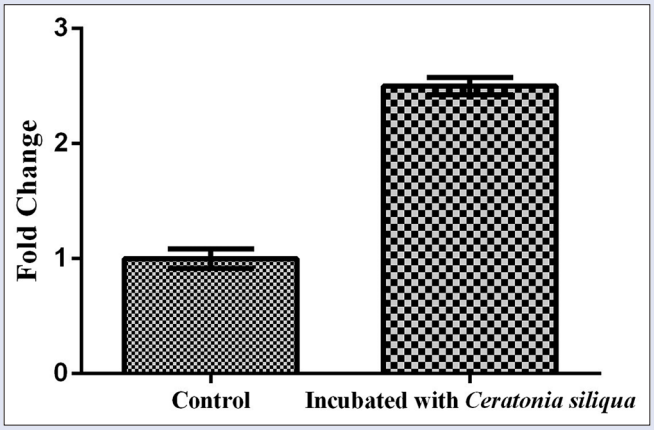

Figure 2: Effects of *Ceratonia siliqua* extract on NR3C4 gene expression

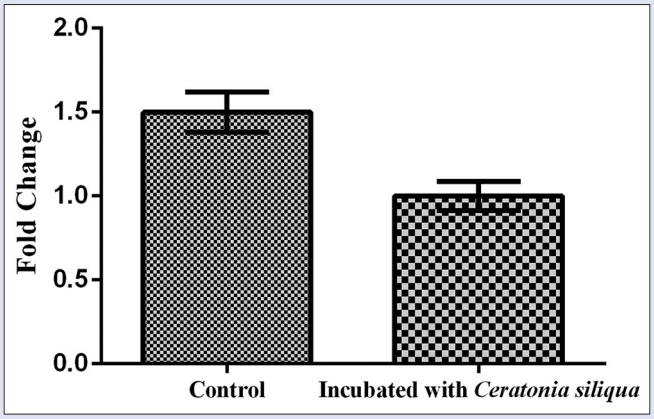

Figure 3: AKT1 gene expression results affected by *Ceratonia siliqua* extract

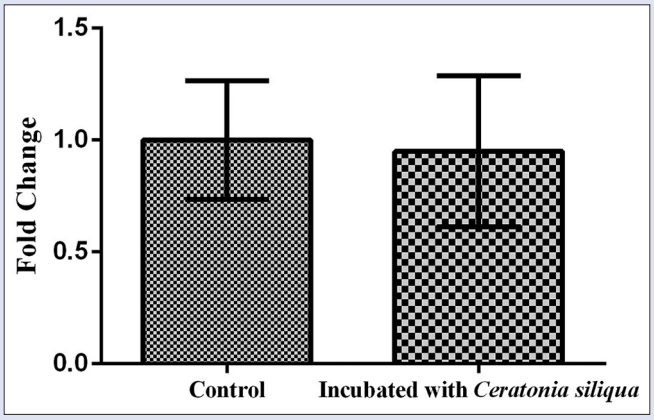

Figure 4: Effects of *Ceratonia siliqua* extract on FUS gene expression. The extract incubation did not change FUS gene expression levels in HEK293 cells

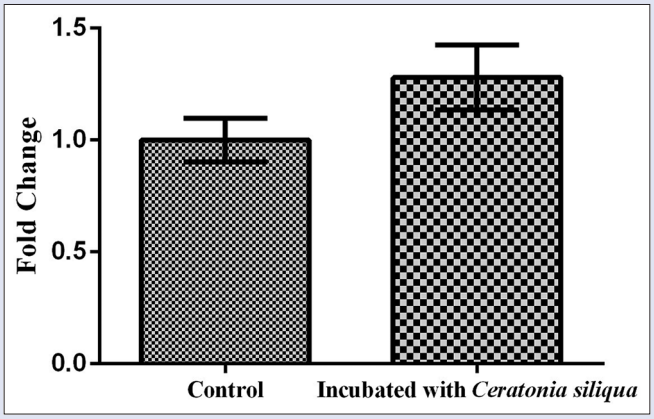

Figure 5: Increased p38 gene expression levels in HEK293 cells by *Ceratonia siliqua* extract

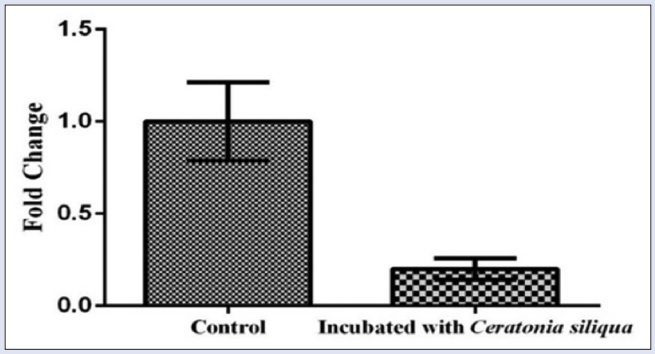

Figure 6: *Ceratonia siliqua* extract decreased kynureninase gene expression in HEK293 cells

gene expression in HEK293 cells. In addition, it changed expression levels of the gene regulating NR3C4 receptor. These results support that its regulating role on the receptor and also that it includes the compounds specifically affecting NR3C4 signaling pathways. Therefore, determination of the molecules and their various combinations with each other can contribute to discover new therapeutic agents for the diseases dependent on AR signaling pathways.

CONCLUSION

Our results suggest that *C. siliqua* extract regulating role on the receptor; furthermore, it includes the compounds specifically affecting NR3C4 signaling pathways. In *C. siliqua* extract, determination of potential new agents for AR gene regulation and their various combinations with each other can contribute to discover new therapeutic agents for the diseases dependent on AR signaling pathways.

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Conflicts of interest

There are no conflicts of interest.

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