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Nuclear receptor agonist activity studies on some *Plantago* species and *Scutellaria salviifolia* Benth.: A particular focus on liver x receptor alpha and retinoid x receptor alpha connected with the inflammation process

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ABSTRACT: Nuclear receptors are a superfamily of transcription factors that regulate gene transcription and intracellular function. They have a key role in various biological processes. Liver X receptors (LXR) mainly play role in lipid metabolism, cholesterol synthesis, glucose homeostasis, and inflammation whereas retinoid X receptor alpha (RXRα) plays important role in inflammation and immunity. In this study, *Plantago holosteum, P. major* subsp. *major*, *P. lagopus, P. scabra*, and *Scutellaria salviifolia* were selected for further investigation due to their anti-inflammatory activities. For this purpose, liver x receptor alpha (LXRα) and retinoid x receptor alpha (RXRα) agonist activity of the extracts was determined through luciferase reporter gene assay on HEK293 cells at a concentration of 100 µg/ml. *P. holosteum, P. major* subsp. *major*, *P. lagopus*, and *P. scabra* extracts showed weak LXRα agonist activity with the fold values in a range of 0.95-1.39. *P. major* showed the highest agonist activity among tested *Plantago* species. For LXRα agonist activity, *S. salviifolia* extract had a fold value of 2.37 which was comparable to the positive control T09011317 at 10 nM concentration. Results for RXRα agonist activity found to be similar to LXRα for all extracts. *S. salviifolia* showed the highest RXRα agonist activity with a fold value of 1.62. Results suggested that *Scutellaria* species, which are used as traditional medicine especially in eastern Asia due to its anti-inflammatory effects, might show this effect due to LXRα activation.

KEYWORDS: *Plantago; Scutellaria;* liver x receptor; retinoid x receptor; anti-inflammatory.

1. INTRODUCTION

Nature is a major source for drug discovery studies. Traditional medicine is recognized as one of the health care systems in many countries. This is due to a number of reasons including affordability, accessibility and low cost. In this respect, the flora of Turkey is wealthy. And have great importance in the prevention and treatment of several diseases with various plants. The flora of Turkey comprises about 12,000 vascular plant taxa, of which about 32% are endemics [1]. In this study, species from two genus *Plantago* (Plantaginaceae) and *Scutellaria* (Lamiaceae) were chosen for further investigation due to their anti-inflammatory effects. These plants have been used for their anti-inflammatory effects traditionally in Turkey. *Plantago* genus is comprising about 275 species all around the world [2]. The genus is represented by 21 species in Turkey which 2 of them are endemic. The main uses of *Plantago* species in folk medicine are for their; wound healing, anti-inflammatory, antidiabetic, and anticancer effects [2, 3]. Phytochemical studies performed on the genus showed the presence of mainly iridoid glycosides, phenylethanoid glycosides, terpenoids, steroids, and polysaccharides [2-4]. Aucubin, catalpol, and acteoside are the main characteristic compounds of the genus [5].

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Scutellaria genus is widespread in Europe, America, and East Asia which comprises about 350 species in the world [6]. Genus *Scutellaria* is represented by 15 species in Turkey. *Scutellaria* species are one of the frequently used herbs in Traditional Chinese Medicine and have been used for their effects in inflammation, bacterial and viral infections, hypertension, and cardiovascular diseases. Extracts of *Scutellaria* species or isolated compounds from the genus werereported possessing antiangiogenic, hepatoprotective, anticonvulsant, antioxidant, antimicrobial, antiviral, antihyperglycemic, and antitumor effects [6-14]. Flavonoids, phenylethanoid glycosides, iridoid glycosides, diterpenes, triterpenes, alkaloids, and essential oil were reported as the main constituents of the genus that were thought to be responsible for the therapeutic effects [6, 15-17].

Nuclear receptors are ligand activated transcription factors thatplay role in various metabolic processes [18, 19]. Retinoid X receptors (RXRα, β, γ) are members of the nuclear receptor family. They form homodimers with themselves or heterodimers with other members of the nuclear receptor family [20]. RXRα plays important role in inflammation and immunity suppressing the expression of proinflammatory genes [21, 22]. Retinoic acid receptor (RAR), peroxisome proliferator-activated receptor (PPAR), liver X receptor (LXR), and farnesoid X receptor (FXR) are other receptors in the nuclear receptor family. Among these, liver X receptors were firstly identified in the mid-1990s [20]. LXRs form heterodimers with RXR, and this heterodimer can be activated by a ligand of either partner [18, 23]. Their role in lipid metabolism and other biological processes were shown mostly in the last two decades [19]. Studies showed LXRs have anti-inflammatory effects by repressing inflammatory genes [23]. Repression of genes such as iNOS, COX-2, IL-6, the chemokines monocyte chemoattractant protein-1 (MCP-1) and MCP-3, and matrix metalloproteinase-9 (MMP-9) were reported. It was also reported that LPS stimulated LXR knockout mice resulted in increased inflammatory response with high expression of iNOS, TNF-α, and IL-1β [24].

In this study, *Plantago holosteum, P. major* subsp. *major, P. lagopus, P. scabra,* and *Scutellariasalviifolia* were investigated for their effects on LXR and RXR. As LXR plays a critical role in inflammation, the role of activation of LXR and RXR in anti-inflammatory effects of these plants will be discussed.

2. RESULTS AND DISCUSSION

In this study, *Plantago holosteum, P. major* subsp. *major, P. lagopus, P. scabra,* and *Scutellariasalviifolia,* which were shown to possess anti-inflammatory activity, were examined for their effects on nuclear receptors such as LXRα and RXRα. Extracts were applied at a concentration of 100 µg/ml in each assay. LXRα agonist T09011317, and RXRα agonist bexarotene were used as positive control at the concentrations of 1,10 and 100 nM. The results were given as fold induction values normalized by *β*-galactosidase. *P. holosteum, P. major* subsp. *major, P. lagopus, P. scabra* extracts showed weak LXRα and RXRα agonist activity (Figures 1 and 2).

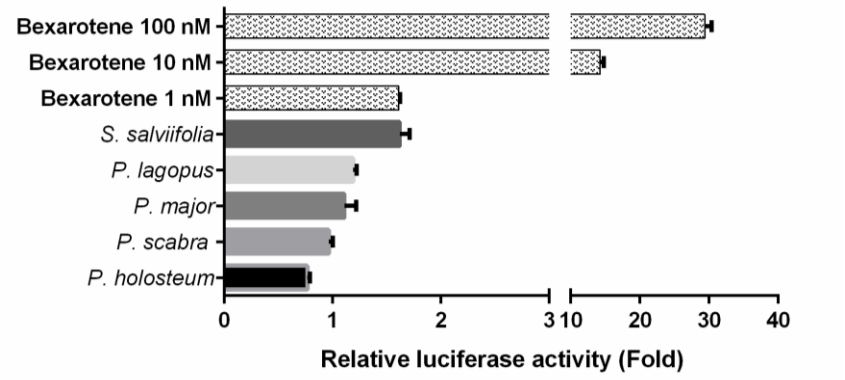

Figure 1. Screening of aqueous extracts obtained from the aerial parts of *S. salviifolia*, *P. lagopus, P. major* subsp. *major, P. scabra,* and *P. holosteum* for retinoid X receptor (RXR) agonist activity using luciferase reporter gene assay at the concentration of 100 µg/ml. Three independent test results were considered, averages and standard error means were calculated and shown in the figure.

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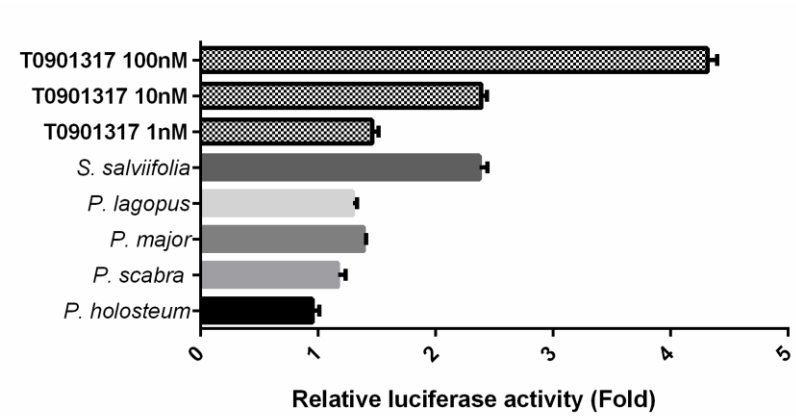

Figure 2. Screening of aqueous extracts obtained from the aerial parts of *S. salviifolia*, *P. lagopus, P. major* subsp. *major, P. scabra,* and *P. holosteum* for liver X receptor (LXR) agonist activity using luciferase reporter gene assay at the concentration of 100 µg/ml. Three independent test results were considered, averages and standard error means were calculated and shown in the figure.

P. holosteum and *P. scabra* had almost no effect on LXRα activation with fold values of 0.95 and 1.17 respectively. *P. major* extract showed higher LXRα agonist activity when compared to the other *Plantago* extracts tested in the study, with a fold value of 1.39 which was similar to the 1 nm concentration of the positive control T0901317. RXRα agonist activity results for tested *Plantago* extracts showed that they had almost no activity (Figure1). Fold values were found in a range of 0.77-1.19. These findings suggested that components of *Plantago* extracts showed activity through LXR-RXR heterodimer as a LXR ligand, not as a RXR ligand.

S. salviifolia extract showed higher agonist activity to both nuclear receptors when compared with tested *Plantago* extracts. *S. salviifolia* showed high LXRα agonist activity with a fold value of 2.37. This value is almost the same as the fold value (2.38) of T0901317 at 10 nM concentration (Figure2). RXRα agonist activity of *S. salviifolia* was also found to be comparable with the positive control bexarotene at 1 nM concentration (Figure 1). These findings suggested that components of *S. salviifolia* extract might show activity through LXR-RXR heterodimer as an LXR ligand, maybe together with synergistic effect through RXR agonist activity.

Plantago species have been known as medicinal plants and widely used in traditional medicine for the treatment of various diseases for many years [2, 25]. In our previous studies, we isolated iridoid glycosides, phenylethanoid glycosides, and a lignan glycoside from *P. holosteum* and phenylethanoid glycosides, and an iridoid glycoside from *P. major* subsp. *major* [26]. We also previously demonstrated that *P. holosteum* exhibited significant inhibitory activity against hyaluronidase and collagenase enzymes leading to the wound healing process [27]. Protective effect of aqueous extract of *P. holosteum* on H_2O_2-induced damaged L929 fibroblasts and anti-inflammatory effect on LPS – induced RAW 264.7 cells by decreasing the level of NO, PGE_2, and TNF-*a* mediators were also determined in our previous studies [3]. Additionally, the aqueous extract of aerial parts of *P. major* subsp. *major* was reported to inhibit hyaluronidase and collagenase enzymes prominently [28]. Furthermore, *in vitro* cytotoxic effects of *P. lagopus* and *P.holosteum* were determined against HEp-2, RD, and MCF-7 cell lines in previous studies [4, 29, 30]. In accordance with our results, anti-inflammatory, wound healing and anticancer effects of these *Plantago* species may not be related to their liver X receptor alpha and retinoid X receptor alpha agonist activity.

Scutellaria species have several usages worldwide because of their anti-inflammatory, antitumor, anticonvulsant, anticancer, and antimicrobial effects [6]. *S. baicalensis* is one of the most popular *Scutellaria* species, clinical studies have been conducted to show its anti-inflammatory properties [31-33]. Its anti-inflammatory activity may be attributed to various flavonoids content [34, 35]. In our previous study, methyl-α-pyrone glucosides, phloroglucinol glucosides, flavonoids, and phenylethanoid glycosides were isolated from *S. salviifolia* [15]. In the present study, one of the selected plants, *S. salviifolia* is an endemic species in Turkey. Because of the usage of genus in traditional medicine, it is valuable for investigation on anti-inflammatory studies. Additionally, aerial parts of *S. salviifolia* is rich inflavonoids and phenolic compounds [15]. In our previous study, the anti-inflammatory activity of aqueous extract from *S. salviifolia* was tested via nitric oxide (NO) and

interleukin-6 (IL-6) cytokines. Concentration depended anti-inflammatory effect was determined at the tested concentration of 50-200 µg/ml. *S. salviifolia* significantly reduced the production of NO and IL-6 at 200 µg/ml compared to positive control LPS alone on LPS stimulated RAW 264.7 macrophage cells (p <0.001) [14]. In accordance with the studies conducted previously on *Scutelleria* species, our findings suggested that LXRα and RXRα activation might have a role in the anti-inflammatory potential of the plant.

3. CONCLUSION

In this study, the potential LXRα and RXRα agonist activities of some *Plantago* and *Scutellaria* spec. extracts were evaluated. The LXRα agonist activity of *S. salviifolia* was found to be comparable with the positive control T09011317 while the agonist activity of tested *Plantago* species werefound to be lower. *Scutellaria* species are widely used as traditional medicine especially in eastern Asia due to their anti-inflammatory effects. As LXRα and RXRα play an important role in inflammation, anti-inflammatory effect of *Scutellaria* species may be due to this pathway.

Although our *in vitro* cytokine- and nuclear receptor-based study results are comparable with the anti-inflammatory effect of *S. baicalensis* and support the literature data, *in vivo* experiments are required to prove anti-inflammatory activity of *S.salviifolia.*

4. MATERIALS AND METHODS

4.1. Cell lines and cell culture media

HEK293 (Human embryonic kidney) cell line was obtained from Riken Bioresource Center Cell Bank (Ibaraki, Japan). Minimum Essential Medium Earle's salts (MEM's Earle) trypsin, sodium dodecyl sulfate, and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma Aldrich (St. Louis, MO, USA). Fetal bovine serum was obtained from Biowest. Penicillin, streptomycin, MEM non-essential amino acids solution were purchased from Wako Pure Chemical Industries (Osaka, Japan). Bexarotene and T0901317 were obtained from Cayman Chemical Co. (Ann Arbor, MI, USA).

4.2. Plant materials

Aerial parts of *Plantago holosteum*, *P. major* subsp. *major*, *P. scabra*, *P. lagopus*, and *Scutellaria salviifolia* were investigated in this research. The collection sites, collection dates, herbarium numbers of the voucher specimens, yields of the extract (given as w/w) were given in Table 1.

Table 1. Detailed information for the plant materials used in the study.

Plant name	Collection site (located in Turkey)	Collection date	Herbarium number for voucher specimen	The specialist that identified the plant material	Yield % (w/w)
P. major subsp. *major* L.	Macka/ Trabzon	June 2008	09009	Dr. Serdar Aslan (Duzce University, Turkey)	20.2
P. lagopus L.	Antalya/ Duden Waterfall	April 2009	09325	Prof. Dr. Zeki Aytaç (Gazi University, Turkey)	15.6
P. scabra Moench	Ankara to Kırıkkale roadsides, near Kırıkkale	June, 2011	11002	Prof. Dr. Zeki Aytaç	13.3
P. holosteum Scop.	Beypazari/Ankara	May, 2011	11001	Prof. Dr. Zeki Aytaç	15.0
S. salviifolia Benth.	Mamak/Ankara	June, 2012	12003	Prof. Dr. Zeki Aytaç	20.9

4.3. Extraction

The same extraction method was used for all plants. The air-dried aerial parts of the plants were extracted with methanol. Methanol was evaporated under vacuum to yield extract. Then it was dissolved in water and partitioned with petroleum ether to remove chlorophylls and other lipophilic compounds. The aqueous fractions were lyophilizedand dissolved in dimethylsulfoxide(DMSO) to obtain stock solutions. Dilutions were made through medium and used in the biological activity tests at a concentration of 100 µg/ml (the final DMSO concentration was 0.1%). The yields obtained for the extracts were given in Table 1.

4.4. Luciferase reporter gene assay

To determine the RXR and LXR agonist activity; NR expression vectors, luciferase reporter plasmids, pCMX-β- gal expression vector, and carrier DNApuc18 were used for transfection [36]. HEK293 (human embryonic kidney cells) were used in the assay. Cells were seeded at 48 well plate at a density of $1x10^5$ cells. Minimum essential medium with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 50 U/ml penicillin, and 50 µg/ml streptomycin was used for cell culture. For LXR luciferase reporter assay, pBApo- CMW-hLXR-α (30 ng), pGL4.1-DR4-Luc (120 ng), pCMX-β- gal expression vector (30 ng), and carrier DNA pUC18 were used to yield a total of 600 ng of DNA per well. For RXR, pCMX-hRXR α (60 ng), TK-CRBP-2-Luc (150 ng), pCMX-β- gal expression vector (30 ng), and carrier DNA pUC18 were used to yield a total of 600 ng of DNA per well. The calcium phosphate co-precipitation method was chosen for transfection. Extracts were applied after 6h and incubated for a 36h time period. After incubation cell lysates were prepared to determine the luciferase and β-galactosidase activities. Results were analyzed after normalization of luciferase activity by β-galactosidase activity [37]. Fold induction values relative to vehicle treated cells were calculated. A known LXRα agonist T0109317 and RXRα agonist bexarotene were used as positive control and vehicle treated cells were used as negative control. Three independent test results were considered, averages and standard error means were calculated and shown in the figure (Figures 1 and 2).

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