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The Effects of Different Concentrations of Foliar Applied Chitosan, Iron Oxide and Chitosan-Coated Iron Oxide Nanoparticles on the Secondary Metabolites of *Hypericum triquetrifolium* Turra. During Full Bloom

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ABSTRACT

Hypericum triquetrifolium Turra. (Hypericaceae) is one of the important medicinal plants. This herb is used in Turkish folk medicine for its antidepressant, anthelmintic and antiseptic effects. Hypericum extracts have an important commercial value in the pharmaceutical industry. Therefore, studies to increase the amount of secondary metabolites it contains are becoming widespread. Elicitors are biological and non-biological factors that can affect the synthesis of secondary metabolites in plants. In recent years, nanoelicitors have been used to increase the amount of active ingredients. In this study, to stimulate the synthesis of biologically active secondary compounds of *H. triquetrifolium*; chitosan, iron oxide and chitosan-coated iron oxide nanoparticles in concentrations of 0 (control), 50, 75, 100 and 150 ppm were sprayed on the leaves during full bloom. LC-MS/MS analysis showed that application of 100 and 150 ppm chitosan nanoparticles increased the amount of flavonol (hyperocyte and quercitrin) and naphthodianthrons (pseudohypericin and hypericin) in *H. triquetrifolium*. 50 ppm iron oxide nanoparticle hyperocyte, quercitrin and pseudohypericin; 75 and 100 ppm iron oxide nanoparticles increased the amount of hyperocyte, quercitrin and hyperforin. The 150 ppm iron oxide nanoparticle resulted in an increase in all compounds except hypericin. In this study, iron oxide nanoparticles coated with chitosan were also used as elicitors to improve the chemical and biological properties of iron oxide. In this series, iron oxide nanoparticle coated with 100 ppm chitosan was effective and increased the amounts of quercitrine, kaempferol and pseudohypericin. The concentration of 75 ppm of this group was effective on quercitrin.

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Tam Çiçeklenme Döneminde Yapraktan Uygulanan Kitosan, Demir Oksit ve Kitosanla Kaplanmış Demir Oksit Nanopartiküllerinin Farklı Konsantrasyonlarının *Hypericum triquetrifolium* Turra.'nın İkincil Metabolitleri Üzerine Etkileri

ÖZET

Hypericum triquetrifolium Turra. (Hypericaceae) önemli tıbbi bitkilerden biridir. Bu bitki Türk halk tıbbında antidepresan, antelmintik ve antiseptik etkileri nedeniyle kullanılmaktadır. Hypericum özütleri ilaç endüstrisinde önemli bir ticari değere sahiptir. Bu nedenle içerdiği ikincil metabolitlerin miktarını artırmaya yönelik çalışmalar yaygınlaşmaktadır. Elisitörler, bitkilerde ikincil metabolitlerin sentezini etkileyebilen biyolojik ve biyolojik olmayan faktörlerdir. Son yıllarda, aktif bileşen miktarını artırmak için nanoelisitörler kullanılmaktadır. Bu çalışmada, *H. triquetrifolium*'un biyolojik aktif sekonder bileşiklerinin sentezini uyarmak için; tam çiçeklenme döneminde yaprakların üzerine 0

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(kontrol), 50, 75, 100 ve 150 ppm konsantrasyonlarında kitosan, demir oksit ve kitosanla kaplanmış demir oksit nanopartikülleri püskürtülmüştür. LC-MS/MS analizi, 100 ve 150 ppm'lik kitosan nanopartikülleri uygulanmasının, *H. triquetrifolium*'da flavonol (hiperosit ve kuersitrin) ve naftodiantronların (psödohiperisin ve hiperisin) miktarını artırdığını göstermiştir. 50 ppm demir oksit nanopartikülü hiperosit, kuersitrin ve psödohiperisin; 75 ve 100 ppm'lik demir oksit nanopartikülleri ise hiperosit, kuersitrin ve hiperforin miktarlarını artırmıştır. 150 ppm'lik demir oksit nanopartikülü, hiperisin dışındaki tüm bileşiklerde artış sağlamıştır. Bu çalışmada, demir oksidin kimyasal ve biyolojik özelliklerini iyileştirmek için kitosanla kaplanmış demir oksit nanopartikülleri de elisitör olarak kullanılmıştır. Bu seride, 100 ppm'lik kitosanla kaplanmış demir oksit nanopartikülü etkili olmuş ve kuersitrin, kaempferol ve psödohiperisin miktarlarını arttırmıştır. Bu grubun 75 ppm'lik konsantrasyonu kuersitrin üzerinde etkili olmuştur.

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INTRODUCTION

Among the many plants used in treatment, Hypericum genus stands out with its antidepressant properties thanks to its hypericin and derivatives content (Acar, 2018). Anatolia is rich in Hypericum species and 46 of the 96 species grown here are endemic. The best known members of the genus are *Hypericum perforatum* L. and *Hypericum triquetrifolium* Turra. These species are known among the locals by names such as kantaron, binbirdelik otu, kan otu, kılıç otu, yara otu and kuzukıran (Baytop, 1999).

In addition to its antidepressant properties, Hypericum extracts are also used in the treatment of many diseases such as wound healing, diabetes, rheumatism, stomach and intestinal ulcers, lymphatitis, mumps and hepatitis (Baytop, 1999; Süntar et al., 2016; Mullaicharam and Halligudi, 2019). Hypericin and hyperforin obtained from Hypericum species have been determined to cause cell death in different cancer types (Agostinis et al., 2002). Hypericum species contain a large number of secondary metabolites including naphthodiantrons, floroglusinol, flavonoids, various organic acids, essential oils, xanthons, tannins and other water soluble components (Oskay and Oskay, 2009).

Increasing the amount of secondary metabolites, produced in small quantities during the plant's specific developmental stage or stress, is one of the most important issues recently studied.

Various applications are made to increase the amount of secondary metabolites in plants. Among these, the use of different elicitors stands out. Elicitors that cause various physiological changes in the target living organism can be of biotic or abiotic origin. In recent years, nano elicitors (size range of 1 and 100 nm) have been used since they are taken more easily by the plant and have a quicker effect.

Chitosan, one of the nano elicitors used in the study, is a biopolymer obtained by partial deacetylation of the chitin (Muxika et al., 2017). Chitosan application is accepted as an effective strategy to increase the production of xanthones and other polyphenols in *H. perforatum* in vitro fringe root cultures (Tocci et all.,2010 and 2011; Brasili et al., 2014). Chitosan has been proven to promote plant growth, maintain the safety of edible products and stimulate abiotic and biotic stress tolerance in various horticultural crops (Abdel-Aziz et al., 2018).

Nano metal oxides, which have started to be produced especially to increase the yield and quality of herbal products, will be an important technology branch in the future (Dağhan, 2017; Brunner et al., 2006). Nano metal oxides, which are easily taken by the plant, have unique effects such as increase in production, increase in photosynthesis and significant expansion in leaf surface area (Abdel-Aziz et al., 2018).

Inorganic compounds such as metal oxides can act as chemical elicitors of plant secondary metabolism (Trillini et al., 2006). Zinc and iron nano oxides have been found to stimulate the production of secondary metabolites in *H. perforatum* cell suspension culture (Sharafi et al., 2013).

Iron oxide nanoparticles are widely used due to its properties such as biocompatibility, non-toxicity, water solubility and low cost compared to many other metallic nanoparticles (Askary et al., 2017; Nehra at al., 2018).

Coating with organic polymers can improve the physical, chemical and biological properties of metal oxide nanoparticles (Zhu et al., 2018). Chitosan is a widely used organic polymer for this purpose (Kumar et al., 2000; Rabea et al., 2003).

In addition, coating with chitosan allows metal oxide nanoparticles to enter plants more easily (Abdel-Aziz et al., 2018). For this reason, the effect of chitosan-coated iron oxide nanoparticle on the biologically active compounds of *H. triquetrifolium* was also investigated during full bloom.

MATERIAL and METHOD

Plant Material

H. triquetrifolium seeds were collected from the Dicle University campus in 2018. The species was identified by Dr. A. Selçuk Ertekin. Herbarium specimens are preserved in the Herbarium of the Faculty of Science Biology Department (DUF 2512-a)

H. triquetrifolium is a 15-77 cm tall perennial plant containing thin hairs (Davis, 1966). It has yellow flowers with a 5-piece petal. The plant is perennial and has a fringe root system. The leaves are mutually arrayed on the stem (Figure 1).

Figure 1. Flowers of *H. triquetrifolium*
Şekil 1. *H. triquetrifolium*'un çiçekleri

Germination of H.*triquetrifolium*

H. triquetrifolium seeds were stratified by keeping in the refrigerator at +4°C for 15 days. Then the seeds were placed in pots and left in the plant growing room at the alternating temperatures +15°C/25°C with a 8 hours dark/16 hours light period. Irrigation has been applied during this period. The seeds started to germinate after about 20 days in this conditions.

Preparation of Solutions

The chitosan, iron oxide and chitosan-coated iron oxide nanoparticles used in this study, were synthesized and defined by Dr. Bilsen Tural and Erdal Ertaş at the labratory of Chemistry Department at Ziya Gökalp Faculty of Education, Dicle University (Tural et al., 2016).

Preparation of chitosan nanoparticle solution

Chitosan solutions were prepared in 10% acetic acid solution at concentrations of 50, 75, 100 and 150 ppm.

Preparation of iron oxide (Fe_3O_4) nanoparticle solution

Iron oxide nanoparticles were weighed on precision scales and dispersed in water at 50, 75, 100 and 150 ppm concentrations.

Preparation of chitosan-coated iron oxide nanoparticle solution

The chitosan-coated iron oxide nanoparticles were weighed on a precision scale and dispersed in water at concentrations of 50, 75, 100 and 150 ppm.

Nanoparticle Application on the Leaf

Nanoparticle applications were made by spraying the leaves of the plants. The nanoparticle disperses were thoroughly mixed with vortex just before spraying. 0 (control), 50, 75, 100 and 150 ppm chitosan, iron oxide and chitosan-coated iron oxide complex nanoparticle disperses were prepared and sprayed on the above ground parts of the plants separately. Distilled water was used for the control group. Plants were harvested 10 days after application since the most effective harvest day were 7-14 days in cell culture studies (Simic, et al.,2015).

Extraction of Phenolic Compounds

By adding 10 mL chloroform to the air dried and grinded 0.2 g aerial parts of plant sample was sonicated for 20 minutes with Sanyo MSE-Soniprep 150, U.K. Samples were centrifuged at 5000 rpm for 5 minutes. 100 microliters from the supernetant was taken and completed to 100 microliters with methanol and left in the vials and stored in the refrigerator until LC-MS/MS analysis.

LC-MS / MS Device

Secondary compound analysis was done by Shimadzu LCMS 8040 (LC-MS / MS system Shimadzu Nexera model UHPLC).

It consists of LC-30 AD model gradient pump, DGU-20A3R model degaser, CTO10ASvp model column furnace and SIL-30AC model auto sampler. Chromatographic separation was performed on the

Agilent Poroshell 120 (EC-C18 2.7 µm, 4.6 mm × 150 mm) column. LC-ESI-MS / MS data were collected and processed with LabSolutions (Shimadzu, Kyoto, Japan) software registered on the instrument.

Statistical Analysis

Statistical analysis were performed using SPSS 15.0. Significance was determined by analysis of variance (ANOVA) and comparisons for the groups were made with Duncan's multiple range test. The level of significance was defined as $P \leq 0.05$.

RESULT and DISCUSSION

H. triquetrifolium is a widely used Hypericum species for its antidepressant, antihelmintic and antiseptic effects in Turkey (Baytop 1999). Various studies have been carried out to increase the amount of hypericin and hyperforin in cell cultures by using various elicitors. Whether the amounts of some secondary compounds change by applying nano oxide elicitors to the leaves of Hypericum species has not been the subject of research.

Using the previous studies (Sharafi et al., 2013; Ahmad et al., 2018), 0 (control), 50, 75, 100 and 150 ppm chitosan, iron oxide and chitosan coated iron oxide complex nanoparticle disperses were prepared and sprayed on the aboveground parts of the plants during full bloom. Plants were harvested 10 days after application since the most effective harvest day for phenil propanoids were 7-14 days in cell culture studies (Simic et al., 2015).

The secondary metabolite amounts of H. triquetrifolium in various growth stages collected from natural environments have been the subject of various studies (Hosni et al., 2011; Alali et al. 2004). The secondary metabolite contents of the control group plants in our study were generally close to previous studies. In a study investigating the amount of some metabolites of H. triquetrifolium grown in natural environment during the flowering period, hypericin was determined as 0.10 mg ml-1 , pseudohypericin 0.12 mg ml-1 , hyperoside 17.6 mg ml-1 and quercitrin 10.44 mg ml-1 (Hosni et al.,2011).

In our study, among these compounds, hypericin 0.31 mg ml-1 , pseudohypericin 0.37 mg ml-1 , hyperocyte 14.61 mg ml-1 seem to be consistent with other findings, while the amount of quercitrine was found to be low (0.50 mg ml-1).

Again Alali et al., in 2004, they determined the hypericin content in H. triquetrifolium as (0.36 mg ml-1). This result seems to agree with our data in the control group (0.31 mg ml-1).

Plants produce a variety of secondary metabolites known to function to protect against living or non-living agents. Elicitors can activate defense systems by stimulating the metabolic processes of target plants. The use of signal molecules as elicitors provides an efficient technique for the production of secondary compounds in plants (Hatami et al., 2018).

The Effects of Chitosan Nanoparticles

LC-MS / MS analysis showed that application of 100 ppm and 150 ppm chitosan nanoparticles increased the amount of flavonol (hyperocyte and quercitrin) and naphthodianthrons (pseudohypericin and hypericin) in H. triquetrifolium. Hyperforin, a phloroglucinol, did not respond to any concentration of the chitosan nanoparticle.

100 ppm chitosan applied from the leaf during full blooming; increased the amount of hyperocyte (18.42±0.52 mg ml-1), quercitrine (0.70±0.02 mg ml-1), kaempferol (0.21±0.01 mg ml-1) and pseudohypericin (0.43±0.01 mg ml-1) compared to control (14.61±0.42; 0.50±0.01; 0.11±0.00; 0.37±0.01 mg ml-1 respectively). In this period, 150 ppm chitosan; caused an increase in the amounts of hyperocyte (19.33±0.56 mg ml-1), quercitrine (0.87±0.03 mg ml-1), pseudohypericin (0.46±0.01 mg ml-1) and hypericin (0.36±0.01 mg ml-1). The highest amount of kaempferol (0.21±0.01 mg ml-1) was obtained from a concentration of 75 ppm (Table 1).

Chitosan is used as an elicitor to stimulate the production of pharmaceutically useful compounds in both plant and in vitro systems. (Lei et al., 2011; Yin et al., 2012; Bistgani et al., 2017).

There is no study on the effect of foliar applied chitosan on the secondary metabolites of H. triquetrifolium. However, the effect of chitosan applied to cell and tissue culture media on the secondary metabolites of some Hypericum species were studied. Chitosan stands out as a polysaccharide elicitor that increases the amount of of xanthones and other polyphenols in cell cultures of H. perforatum (Simic et al., 2015).

Foliar application of nanoparticles increases the productivity and quality of the products as it enables the nutrients to enter the plant system directly (Mahil et al., 2019).

Application of 100 ppm and 150 ppm chitosan nanoparticles increased the amount of flavonol (hyperocyte and quercitrin) and naphthodianthrons (pseudohypericin and hypericin) in H.triquetrifolium. Hyperforin, a phloroglucinol, did not respond to any concentration of the chitosan nanoparticle (Table1).

Here, it has been suggested that, chitosan stimulates the defense system by giving the sensation of pathogen attack and provides the synthesis of some plant defense compounds (Franklin et al. 2009).

The Effects of Iron Oxide Nanoparticles

50 ppm iron oxide nanoparticle increased the amount of hyperocyte (14.58±0.42 mg ml-1), quercitrin

(0.67±0.02 mg ml-1) and pseudohypericin (0.42±0.01 mg ml-1). 75 ppm and 100 ppm iron oxide nanoparticles increased the hyperocyte, quercitrin and hyperforin amounts. 150 ppm iron oxide nanoparticle caused a statistical increase in all compounds except hypericin. (Table 2).

It has been found that iron oxide nanoparticles greatly increase the total phenolic, flavonoid and some polyphenol content in some plants (Marslin et al., 2017; Nourozi et al., 2019).

Different concentrations of iron oxide nanoparticles affected the secondary metabolites of H. triquetrifolium in different ways. Hyperocyte and quercitrine responded to all concentrations of iron oxide nanoparticles and showed statistically significant changes compared to control. Both compounds showed maximum amounts at a concentration of 150 ppm. Hyperforin, a phloroglucinol derivative, yielded the highest amount at 75 ppm iron oxide nanoparticle concentration.

Pseudohypericin (naphthodiantrone) showed maximum amounts at concentrations of 50 ppm and 150 ppm of iron oxide nanoparticle. Hypericin did not increase at any concentration of iron oxide nanoparticle relative to control (Table 2.).

In a study, it was shown that the hypericin and hyperforin content of cell suspension cultures of H. perforatum treated with iron nano oxide (50, 100 and 150 µg l-1) increased compared to control (Sharafi et al., 2013). In our study, while hypericin was not affected by any iron oxide nanoparticle concentrations, pseudohypericin was affected by concentrations of 50 ppm and 150 ppm and increased compared to control.

In another study, 100 µmol iron oxide nanoparticle applied to the nutrient medium under in vitro conditions significantly increased the amount of flavonoid in H. perforatum (Masjedlo and Mahtab 2020). In our study, the quercitrin and kaempferol increased in all concentrations of iron oxide nanoparticles. These findings are seen in agreement with the results we obtained from our study.

The Effects of Chitosan-Coated Iron Oxide Nanoparticles

In this series, concentration of 100 ppm chitosan coated iron oxide nanoparticles was effective and increased the amount of quercitrin (1.07±0.03 mg ml-1), kaempferol (0.24±0.01 mg ml-1) and pseudohypericin (0.43±0.01 mg ml-1). The 75 ppm concentration of this group was effective on quercitrin (0.54±0.02 mg ml-1) (Table 3).

Coating metal oxide nanoparticles with organic polymers can improve their physical, chemical and biological properties (Zhu et al., 2018; Bharathi et al., 2019).

Chitosan is a widely used organic polymer due to its non-toxic, non-easily degradable, antimicrobial activity and biocompatibility properties (Kumar et al., 2000; Rabea et al., 2003). On the other hand, metal oxide nanoparticles coated with chitosan may be easier to take into plants (Abdel-Aziz et al., 2018).

Therefore, the effect of chitosan coated iron oxide nanoparticle on the phenolic compounds of H. triquetrifolium was also investigated.

In this study, a concentration of 100 ppm chitosan coated iron oxide nanoparticles was effective and increased the amount of quercitrin, kaempferol, and pseudohypericin. The 75 ppm concentration of this group was effective only on quercitrin.

Although it is thought that better results can be obtained due to the improvement of the biological and chemical properties of metal oxides coated with chitosan, parallel findings were not obtained in our study.

Table1. The effects of different concentrations of foliar applied chitosan nanoparticles on the secondary metabolites of H. triquetrifolium during full bloom.

Çizelge1. Tam çiçeklenme döneminde yapraktan uygulanan kitosan nanopartiküllerinin farklı konsantrasyonlarının H. triquetrifolium'un ikincil metabolitleri üzerine etkileri.

Compound mg ml^{-1}	Control	50 ppm	75 ppm	100 ppm	150 ppm
Hyperocyte	14.61±0.42^{a}	18.03±0.52^{b}	18.19±0.39^{b}	18.42±0.52^{b}	19.33±0.56^{c}
Quercitrin	0.50±0.01^{a}	0.69±0.02^{b}	0.59±0.02^{c}	0.70±0.02^{b}	0.87±0.03^{d}
Kaempferol	0.11±0.00^{a}	0.18±0.01^{b}	0.19±0.01^{b}	0.21±0.01^{c}	0.19±0.01^{b}
Hyperforin	4.29±0.12^{a}	2.93±0.08^{b}	3.23±0.09^{c}	1.78±0.05^{d}	4.30±0.12^{a}
Pseudohypericin	0.37±0.01^{a}	0.37±0.01^{a}	0.30±0.01^{b}	0.43±0.01^{c}	0.46±0.01^{d}
Hypericin	0.31±0.01^{a}	0.27±0.01^{b}	0.26±0.01^{b}	0.31±0.01^{a}	0.36±0.01^{c}

Each data is the average of three replicates. Differences between the means indicated by different letters in the same line are significant ($P < 0.05$).

Table 2. The effects of different concentrations of foliar applied iron oxide nanoparticles on the secondary metabolites of H. triquetrifolium during full bloom.

Çizelge 2. Tam çiçeklenme döneminde yapraktan uygulanan demir oksit nanopartiküllerinin farklı konsantrasyonlarının H. triquetrifolium'un ikincil metabolitleri etkileri.

Compoundmg ml^{-1}	Control	50 ppm	75 ppm	100 ppm	150 ppm
Hyperocyte	14.61±0.42^{a}	14.58±0.42^{a}	14.09±0.39^{a}	18.03±0.52^{b}	20.50±0.59^{c}
Quercitrin	0.50±0.01^{a}	0.67±0.02^{b}	0.61±0.02^{c}	0.71±0.02^{d}	0.72±0.02^{d}
Kaempferol	0.11±0.00^{a}	0.17±0.00^{b}	0.14±0.00^{c}	0.17±0.00^{b}	0.20±0.01^{d}
Hyperforin	4.29±0.12^{a}	3.22±0.09^{b}	5.36±0.15^{c}	5.26±0.15^{c}	5.17±0.15^{c}
Pseudohypericin	0.37±0.01^{a}	0.42±0.01^{b}	0.34±0.01^{c}	0.37±0.01^{a}	0.42±0.01^{b}
Hypericin	0.31±0.01^{a}	0.28±0.01^{b}	0.24±0.01^{c}	0.31±0.01^{a}	0.28±0.01^{b}

Each data is the average of three replicates. Differences between the means indicated by different letters in the same line are significant (P <0.05).

Table 3. Effects of different concentrations of foliar applied chitosan-coated iron oxide nanoparticles on the secondary metabolites of H. triquetrifolium during full bloom.

Çizelge 3. Tam çiçeklenme döneminde yapraktan uygulanan kitosanla kaplanmış demir oksit nanopartiküllerinin farklı konsantrasyonlarının H. triquetrifolium'un ikincil metabolitleri üzerine etkileri.

Compound mg ml^{-1}	Control	50 ppm	75 ppm	100 ppm	150 ppm
Hyperocyte	14.61±0.42^{a}	10.87±0.31^{b}	12.69±0.37^{c}	14.88±0.43^{a}	12.15±0.35^{c}
Quercitrin	0.50±0.01^{a}	0.45±0.01^{b}	0.54±0.02^{c}	1.07±0.03^{d}	0.46±0.01^{b}
Kaempferol	0.10±0.00^{a}	0.12±0.00^{b}	0.15±0.00^{c}	0.24±0.01^{d}	0.12±0.00^{b}
Hyperforin	4.49±0.12^{a}	2.65±0.08^{b}	3.64±0.10^{c}	2.80±0.08^{b}	4.52±0.13^{a}
Pseudohypericin	0.38±0.01^{a}	0.34±0.01^{b}	0.38±0.01^{a}	0.43±0.01^{c}	0.37±0.01^{a}
Hypericin	0.31±0.01^{a}	0.20±0.01^{b}	0.26±0.01^{c}	0.30±0.01^{a}	0.20±0.01^{b}

Each data is the average of three replicates. Differences between the means indicated by different letters in the same line are significant (P <0.05).

CONCLUSION

According to our results, it can be argued that chitosan stimulates the defense system by giving the feeling of pathogen attack and provides the synthesis of some plant defense compounds. While iron oxide nanoparticles were successful in increasing the amount of quercitrin and kaempferol, it was thought that better results could be obtained due to the improvement of the biological and chemical properties of metal oxides coated with chitosan, which did not reflect positively on the results.

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Statement of Conflict of Interest

Authors have declared no conflict of interest.

Author's Contributions

The contribution of the authors is equal.

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