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Antimutagenic and antimicrobial activity against pathogenic bacteria of endemic *Onobrychis tournefortii* (Fabaceae) fruit extract and its anatomical characterisation

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ABSTRACT:
In this study, the anatomical definition, antimicrobial activity and antimutagenic effects of *Onobrychis tournefortii* were investigated. The antibacterial and antifungal activity of methanolic fruit extract of *O. tournefortii* (OtMFE) were investigated against various pathogen Gram (+) and Gram (-) bacteria and yeast. The findings demonstrated that this extract significantly inhibited the growth of several tested pathogenic microorganisms to varying degrees. The genotoxic effects in human lymphocyte cells *in vitro* were also assessed and confirmed by the micronucleus assay results. In addition, this study showed that *O. tournefortii* exhibits antigenotoxic activity against the mutagenicity of aflatoxin B_1 (AFB_1). This is the first report demonstrating that (OtMFE) has antigenotoxic activity against AFBı-induced DNA damage *in vitro* human lymphocyte cells using the micronucleus (MN) assay. The anatomical features of the fruit of the species, particularly the presence of parallel three-ellipsoid outlines, and the presence of macrosclerides and one-row osteosclerides in the seed coat can be counted among the distinguishing features of the species. This study is the first to investigate the anatomical, antimicrobial and antigenotoxic properties of the Turkish endemic *O. tournefortii* fruit, contributing to both knowledge about the genus and the development of pharmacological products.

Keywords: biological activities, microorganisms, plant anatomy, legume, mutagenicity

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INTRODUCTION

Fabaceae is one of the largest dicotyledonous families, comprising 730 genera and approximately 19,500 species, with numerous economically important plants (Simpson 2006). The genus *Onobrychis* includes 170 species predominantly distributed across the Anatolian-Caucasian triangle, Southwest Asia, the Mediterranean region, and temperate regions of Europe and Asia (Aktoklu 2001). *Onobrychis* species are also well distributed in Turkey with 28 of them classified as endemic (Aktoklu 2001; Yildirimli 2004).

Substances which can cause damage to genetic material are referred to as genotoxins, and are categorised as mutagens, carcinogens, or teratogens based on their mode of action. Genotoxins play a role in the development of various chronic degenerative diseases, including liver disorders, neuro-

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degenerative conditions, cardiovascular diseases, diabetes, arthritis, cancer, chronic inflammation, and aging. In recent years, scientists have discovered new bioactive plant compounds capable of counteracting the harmful effects of physical and chemical mutagens (Izquierdo-Vega *et al*. 2017). Plants provide numerous benefits to humanity thanks to the secondary metabolites they carry (Aruoma 1998). *Onobrychis* species carry flavonoid, folic acid (vitamin B9), ascorbic acid, phenol, carotenoid, phenyl acrylic acid and phenyl methanoic acid as secondary metabolites (Steinmetz & Potter 1996; McCall & Frei 1999).

The growing interest in natural antioxidants has recently highlighted the significance of phytochemicals derived from plant materials, which play an active role in many biological processes. Although some *Onobrychis* species have been investigated for their biological properties, including antibacterial, cytotoxic, antidiabetic and antioxidant activities (Zengin *et al*. 2015; Ozbek *et al*. 2019), to our knowledge, there have been no previous studies on the antimicrobial, antigenotoxic and mutagenic activities of *Onobrychis tournefortii* (Willd.) Desv.

Previous research has revealed that the *Onobrychis* genus may possess antioxidant properties which contribute to its other biological effects. Hence, the antigenotoxic and antimicrobial activities of *O. tournefortii* extracts may prove helpful in the treatment of diseases and the development of new drugs. To date, anatomical studies have mainly focused on the root and stem, and less often on the fruit and seeds (Tekin & Yilmaz 2015; Uzun *et al*. 2020). The aim of this study was to provide a detailed investigation of the anatomical characteristics, antimicrobial activities and antigenotoxic features of the *O. tournefortii* species in Turkey. The findings of the study, presented for the first time, will serve as a valuable resource for future studies on this species.

MATERIALS AND METHODS

The *O. tournefortii* samples selected for this study were collected from Kırşehir (Turkey) in 2019. The collection took place in May and June from the coordinates N 39°10′0.86″, E 34°26′15″. The samples were further processed using alcohol and deposited in a private herbarium (private Herbarium voucher: No. 361, Alcohol stock number: 50). The species were identified according to Davis (1972).

Preparation of the extract. A soxhlet extractor (ISOPAD, Heidelberg, Germany) was used to extract the fruit (100 g) with 1 L of methanol for 72 hours at a temperature below the solvent's boiling point. Following filtration using Whatman filter paper (no. 1), the extract was concentrated under vacuum at 60°C using a rotary evaporator (Buchi Labortechnik AG, Flawil, Switzerland). The plant extract was then lyophilised and stored at +4°C until testing.

Detection of the antimicrobial activity. The antibacterial and antifungal activities of OtMFE (20 mg/ml) against the pathogenic Gram positive bacteria (*Staphylococcus epidermidis* ATCC 12228, *Bacillus cereus* RSKK 863, *Staphylococcus aureus* ATCC25923, and *Micrococcus luteus* ATCC 9341) and Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 700603, *Salmonella typhi* NCTC 9018394, *Proteus vulgaris* RSKK 96026, *Escherichia coli* ATCC 1280, and *Enterobacter aerogenes* ATCC 51342) and yeast (*Candida albicans* Y-1200-NIH were analysed using the well diffusion method (Sari *et al*. 2013). For the extract control, methanol was employed as the solvent, and was found to have no antibacterial action against any of the microorganisms examined. In sterile petri dishes, 1% (v/v) of a 24-hour broth culture (ten bacteria and one yeast) containing 10^6 CFU/mL was

applied. Mueller-Hinton Agar (MHA) (15 mL) was then poured onto the plate, allowed to cool to 45°C, and solidified. After carefully opening wells of 6 mm in diameter and adding with 20 µL/ml of OtMFE, the plates were placed in an incubator and incubated at 37°C for 24 hours.

Following the allotted incubation period, the average value from two trials was utilised to calculate the clear inhibitory zones around each pathogenic yeast and bacterium. Kanamycin (K30 (30µg)), Sulfamethoxazole (SXT25 (25µg)), Ampicillin (AMP10 (10µg)), Amoxicillin (AMC30 (30µg)), Nystatin (NYS100 (100 µg)) were used as the positive controls (Şakiyan *et al*. 2014; Çinarli *et al*. 2019).

The *in vitro* micronucleus (MN) test. For the micronucleus (MN) analysis, peripheral blood samples were obtained from healthy men and women aged 23-25, who did not smoke or drink alcohol, were free of infectious diseases, and not exposed to any physical agents such as X-rays. A total of 0.5 mL of heparinized whole blood was cultured in 7 mL of chromosome medium B (Biological Industries, Beit Haemek, Israel) containing 15% heat-inactivated fetal bovine serum [FBS, (Biochrom, Berlin, Germany)], 1% streptomycin (Gibco, MD, USA), 1% penicillin (Gibco, MD, USA), 2% glutamine (Sigma, MO, USA) and 2% phytohemagglutinin (Biological Industries, Beit Haemek, Israel) (Nartop *et al*. 2020). Aflatoxin B_1 (AFB_1) (Sigma, MO, USA) (5 µM) alone, OtMFE alone (160 µg/mL) and a cotreatment of AFB_1 (5 µM) and different concentrations (120, 160, 240 and 320 µg/mL) of OtMFE were added to this solution and cultured at 37°C for 72 h in a 5% CO_2 moist atmosphere (Nartop *et al*. 2020). AFB_1 was used as the positive control and pure water was used as the negative control. The following steps were followed to establish the experimental setup:

Culture 1: Control
Culture 2: AFB_1 (5 µM)
Culture 3: OtMFE (160 µg/mL)
Culture 4: AFB_1 (5 µM) + OtMFE (120 µg/mL)
Culture 5: AFB_1 (5 µM) + OtMFE (160 µg/mL)
Culture 6: AFB_1 (5 µM) + OtMFE (240 µg/mL)
Culture 7: AFB_1 (5 µM) + OtMFE (320 µg/mL)

For the determination of micronuclei, the procedure previously described by Fenech (2000) was used. 44 h after the initiation of incubation at 37°C, cytochalasin-B (Sigma, MO, USA) was added to each tube at a final concentration of 3 µg/mL to prevent cytoplasmic division. After 72 h, the cells were removed from the incubator and centrifuged at 1000 rpm for 10 min. The supernatant was discarded, a hypotonic solution (6 mL -0.075 M KCl) was added and the mixture was then incubated for 7 min. The cells were then immediately centrifuged and fixed three times with cold methanol / glacial acetic acid (3:1). The fixed cells were dropped onto slides and allowed to dry at room temperature (72 h). The preparations were stained with 6% giemsa (Merck, Darmstadt, Germany) for 10 min. For MN analysis, bi-nucleated cells were evaluated under a light microscope (magnification 1000x) and scored (Nartop *et al*. 2020). Only binucleated cells were considered for MN analysis. For each subject, at minimum of 2000 binucleated cells were analysed for the presence of micronuclei. For the MN scoring, the criteria as described by Countryman and Heddle were followed: a diameter of less than 1/3 of the main nucleus, non-refractility, not touching, and of the same colour as the nucleus, or lighter (Ceker 2021). All procedures were conducted in accordance with the Declaration of Helsinki, approved by the Commission on Ethics and Academic Unity of the Institute of Biodiversity and Ecosystem Research. All donors were informed about the experimental procedure and provided written informed consent.

Anatomical methods. Cross-sections were taken from the fruit of the species by hand. The sections were made into permanent preparations according to the glycerol gelatine method (Vardar 1987). The cell types obtained from the fruit of the species were determined by using the SOIF BK500-L and Amscope MU1803-HS camera imaging system and were photographed (Ulcay 2023). Cell measurements were taken from cross-sections of the species. Anatomical analysis was conducted using 20 plant specimens with an average of 25 measurements taken from the tissues. Measurements were recorded for the epidermis, cuticle, sclerenchyma, sclereids, macrosclereids, and parenchyma cells.

Statistical analysis. To ensure the reliability of the data in this investigation, three replicates were performed for each experimental group. One-way analysis of variance in SPSS version 18.0 was used to analyse the data. Duncan's test was used to establish significance. For all statistical analyses, $p<0.05$ was taken as the significance level.

RESULTS

Antimicrobial potential. The antimicrobial activity (antibacterial and antifungal) of OtMFE was evaluated against four human pathogenic Gram (+) and six Gram (-) bacteria and one yeast. The extract exhibited varying degrees of inhibitory activity (12 mm-25 mm) on the growth of different pathogenic bacteria and yeast when a concentration of 20 mg/ml was used. The antibacterial and antifungal effects of OtMFE were compared with four commercial antibiotics and one anticandidal (Table 1).

Onobrychis tournefortii (OtMFE) showed moderate inhibitory activity against *M. luteus* (15 mm), *S. epidermidis* (12 mm), and *S. aureus* (21 mm), which was lower than that of standard antibiotics. However, this extract showed higher activity in *B. cereus* (22 mm) than AMC (20 mm).

OtMFE exhibited significantly higher inhibition activity than all standard antibiotics in *P. aeruginosa* (25 mm). The genus *Pseudomonas* is widespread in nature, causing opportunistic and nosocomial infections. *Pseudomonas aeruginosa septicemia,* in particular, is frequently observed in debilitated and immunocompromised patients, with a reported mortality rate of 10–20% (Nadaroglu *et al*. 2020) (Table 1).

Salmonella serovars can induce a range of clinical symptoms, from asymptomatic infection to severe typhoid-like syndromes, particularly in infants or extremely vulnerable animals (Öğütçü *et al*. 2017). OtMFE showed higher inhibitory activity (25 mm) than all standard antibiotics against this significant pathogen *S. typhi* (Table 1).

In *K. pneumonia*, OtMFE demonstrated a similar degree (20 mm) of inhibition activity as the antibiotic SXT (Table 1). In addition, this extract exhibited a higher inhibitory effect than standard antibiotics SXT (18 mm), AMP (10 mm) and AMC (14 mm) against *E. coli* (23 mm) (Table 1). Furthermore, the extract showed higher inhibition activity in *P. vulgaris* (21 mm) than standard antibiotics AMP (17 mm) and SXT (19 mm), while demonstrating the same inhibitory activity as AMC (20 mm). Moreover, in *C. albicans* OtMFE showed a higher inhibitory effect (25mm) than the commercial antifungal (20 mm) (Table 1).

Evaluation of the protective capacity against DNA damage: MN assay. As seen in Table 2, the methanol extracts of *O. tournefortii* showed no genotoxic activity at any of the concentrations tested in the present study. However, all the concentrations tested exhibited antigenotoxic activities. In parallel with the increasing concentrations of OtMFE, its antigenotoxic activity also in-

Table 1. Antimicrobial activities of fruit extract of *Onobrychis tournefortii* (diameter of inhibition zone (mm)).

Microorganisms		Plant extract	AMP 10*	SXT 25	AMC 30	K 30	NYS Methanol 100
Gram (+)	*M. luteus*	15	22	21	25	23	N
	S. epidermis	12	26	25	27	25	N
	S. aureus	21	30	24	30	25	N
	B. cereus	22	23	25	20	28	N
Gram (-)	*E. aerogenes*	-	21	19	20	24	N
	P. aeroginosa	25	8	18	15	14	N
	K. pneumonia	20	21	20	21	23	N
	S. typhi	25	11	17	19	20	N
	E. coli	23	10	18	14	25	N
	P. vulgaris	20	17	19	20	21	N
Yeast	*C. albicans*	25	N	N	N	N	20

Standard reagents (diameter of zone inhibition (mm). SXT25 (Sulfamethoxazole); AMP10 (Ampicillin); NYS100 (Nystatin); K30 (Kanamycin); AMC30 (Amoxycillin); N: not tried.

creased and the most successful results were observed for the 240 µg / mL" applications ($p<0.05$).

Anatomical results. The fruit is circular, with its outline consisting of three ellipsoids. These ellipsoids contained two semi-circular seeds each (Fig. 1A). The exocarp in the outermost part of the fruit comprised polygonal shaped epidermis cells with an average width of 20.49 ± 2.37 µm a length of 26.04 ± 1.74 µm. These epidermis cells were covered with a cuticle layer with an average thickness of 6.46 ± 1.21 µm. Just below the epidermis cells, 2–3 rows of circular shaped sclereid cells were formed with an average width of 18.14 ± 0.87 µm and a length of 21.70 ± 0.58 µm (Fig. 1B). Under the circular shaped sclerenchyma cells, elongated sclerenchyma cells were present (Fig. 1C). Polygonal shaped parenchyma cells were noted in the mesocarp. Similar to the exocarp, 2–3 rows of sclerenchyma cells were observed in certain parts of the mesocarp. Single-layered macrosclereids with an average length of 77.19 ± 1.31 µm were present in the outermost part of the seed coat. Under the macrosclereids, sclerenchyma cells with an average diameter of 13.60 ± 0.53 µm and osteosclereids cells with an average width of 18.14 ± 0.87 µm and an average length of 21.70 ± 0.58 µm were identified (Fig. 1D). The parenchyma surrounding the embryo had an average diameter of 40.81 ± 4.92 µm.

DISCUSSION

In this study, the anatomical, antimicrobial and antigenotoxic properties of *O. tournefortii* fruits, naturally grown in Kırşehir and its surroundings, were investigated.

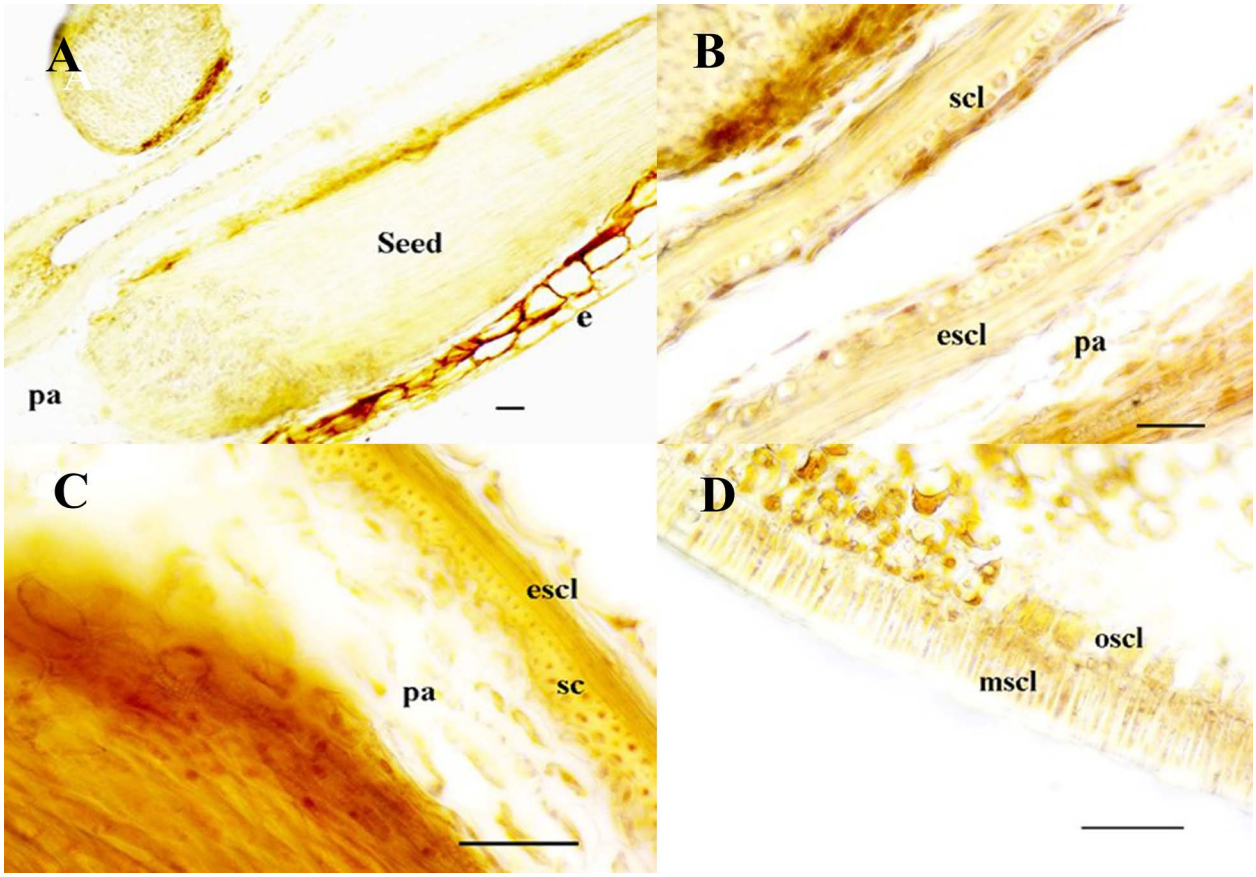

Fig. 1. Fruit and seed image of *Onobrychis tournefortii*. A. Cross-section of seed, B–D cross-section of fruit; epidermis, escl; elongated sclerenchyma cells, sc; sclerenchyma, scl; sclereids, mscl; macrosclereids, oscl; osteosclereids, pa; parenchyma. Scale 100 µm.

Ten bacterial pathogenic strains and one fungal pathogenic strain were tested against OtMFE. The findings revealed that the fruit extract exhibited varying levels of antibacterial activity (12 mm–25 mm) against the examined pathogens ranging from moderate to high, with the extract being significantly more effective against Gram (-) bacteria than against Gram (+) bacteria (Table 1). The presence of an exterior impermeable barrier, a thin peptidoglycan monolayer, periplasmic space, and the composition of the cell wall in Gram-negative bacteria may explain this enhanced activity (Afzal *et al.* 2017). To the best of our knowledge, this study is the first to report the antimicrobial activity of OtMFE, demonstrating high inhibition activity against the tested human pathogenic bacteria and yeast.

According to the findings of Erbil *et al.* (2015), aerial methanolic extracts of *Vicia villosa* Roth, *Trifolium ochroleucum* Huds., and *Onobrychis altissima* Grossh showed no antibacterial activity against the tested microbes. In their study, using the disc diffusion method, they observed that the two weakest inhibitory activities were determined against *S. aureus* (ATCC 25923) and *C. albicans* (ATCC 10231) from the flower methanolic extract and the flower n-hexane extract of *Onobrychis armena* Boiss. & Huet, respectively. Similarly, Bazzaz & Haririzadeh (2003) found that that the methanolic plant extract of *Onobrychis chorassanica* Bunge in Boiss. exhibited no antimicrobial activity and *Onobrychis transcaspica* V. Nikitin demonstrated no antimicrobial activity against *Bacillus subtilis*, *Candida albi*cans, *Escherichia coli*, *Klebsiella pneumonia*, *Morganella morganii*, *Salmonella typhi*, and *Staphylococcus aureus,* while showing insignificant activity against *Pseudomonas aeruginosa*. However, in our study OtMFE showed higher inhibitory activity against *P. aeruginosa S. typhi* and *C. albicans* compared to the standard antibiotics (Table 1).

Table 2 The effects of OtMFE on MN in human peripheral lymphocytes

Test Items	Concentrations	MN numbers ± S.E.
Control (-)		1.72 ± 0.20[a]
Control (+)	5 µM	2.52 ± 0.13[e]
OtME	160 µgr / mL	1.76± 0.32[a]
AFB_{1+} OtME	5 µM + 120 µgr / mL	2.18 ± 0.42[cd]
AFB_{1+} OtME	5 µM + 160 µgr / mL	1.99 ± 0.35[bc]
AFB_{1+} OtME	5 µM + 240 µgr / mL	1.82 ± 0.62[a]
AFB_{1+} OtME	5 µM + 320 µgr / mL	1.94 ± 0.24[b]

AFB^{1} was used as the positive control for human peripheral lymphocytes.a - d, statistically significant differences in the same column are indicated by different superscripts (α = 0.05).

In addition, *O. tournefortii* induced antigenotoxic activity against the mutagenicity of AFB1. This is the first report to show that (OtMFE) exhibits antigenotoxic activity against AFB1-induced DNA damage human lymphocyte cells *in vitro* as assessed using the MN assay.

Although the mechanism underlying the preventive effects of the *O. tournefortii* extract against DNA damage induced by AFB_1 is unclear, this antigenotoxic effect may be attributed to the antioxidant capacity of the extract.

Strong antioxidant activities have been reported in the aerial parts *of O. viciifolia, O. sosnovskyi* Grossh and *O. melanotricha* Boiss (Karamian & Asadbegy 2016). In their study, Karamian & Asadbegy (2016) evaluated the phenolic content and antioxidant activity of the methanolic extracts of three *Onobrychis* species (*O. sosnovskyi* Grossh., *O. viciifolia* Scop. and *O. melanotricha* Boiss.) and found a correlation between antioxidant activities and total phenol content.

No references have been found in the literature regarding the internal structure of the fruit of the species. The fruit of the taxon, classified as belonging to the group of unopened fruits, consists of three ellipsoid outlines. Two similar ellipsoidal outlines were observed previously in *Onobrychis galegifolia* Boiss (Al-Dabbagh *et al*. 2023). Parenchyma-like structures and macrosclereids were observed in the outermost part of *O. tournefortii*, which is in line with previous findings in other *Onobrychis* species (Cenci *et al*. 2000).

CONCLUSION

The development of natural new antimicrobial agents will result in reducing the negative effects of synthetic drugs. Natural antimicrobial agents may be pathogen-specific, biodegradable, antigenotoxic and less toxic to nature. This was demonstrated for the fruit extract of *O. tournefortii*, which can be used as an antimicrobial agent, especially in the treatment of human diseases. The obtained results provide a solid foundation for further research, contributing to both the study of the genus and the development of pharmacological products. Further studies should focus on identifying the active compounds involved in biological activities and exploring any correlations between antigenotoxic activity and antioxidant activities.

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COMPREHENSIVE PLANT BIOLOGY

REZIME

Antimutagena i antimikrobna aktivnost ekstrakata ploda endemične vrste *Onobrichis tournefortii* (Fabaceae) protiv patogenih bakterija i anatomska karakterizacija

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U ovoj studiji su istraživani anatomija, antimikrobna aktivnost i antimutagenost *Onobrichis tournefortii*. Antibakterijska i antifungalna aktivnost metanolnog voćnog ekstrakta *O. tournefortii* (OtMFE) ispitana je protiv nekih patogenih Gram (+) i Gram (-) bakterija i kvasca. Nalazi su pokazali da ovaj ekstrakt značajno inhibira rast nekoliko testiranih patogenih mikroorganizama u različitim stepenima. Pored toga, u trenutnoj studiji procenjeni su genotoksični efekti i na ćelije humanih limfocita *in vitro*. Antigenotoksičnost je pokazana i rezultatima dobijenim iz mikronukleusa. Dodatno, ova studija je pokazala da *O. tournefortii* indukuje antigenotoksičnu aktivnost protiv mutagenosti aflatoksina B1 (AFB1). Ovo je prvi izveštaj da (OtMFE) ima antigenotoksičnu aktivnost protiv oštećenja DNK izazvanih AFB1 in vitro ćelija humanih limfocita korišćenjem mikronukleusnog (MN) testa. Plod vrste, posebno prisustvo paralelnih troelipsoidnih useka u anatomiji ploda, i prisustvo makrosklerida i jednorednih osteosklerida u semenskom omotaču mogu se ubrojati u karakteristične osobine ploda. Anatomska, antimikrobna i antigenotoksična svojstva turske endemične vrste *O. tournefortii* su proučavana prvi put, što će doprineti kako istraživanjima roda, tako i razvoju farmakoloških proizvoda.

Ključne reči: biološka aktivnost, mikroorganizmi, anatomija biljaka, leguminoze, mutagenost