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Antimicrobial activity of leaf extracts of *Bituminaria* sp. genotypes at different growth stages

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Plants have secondary metabolites that play a role in defense mechanism are known to have effects on microorganisms. These metabolites and their amounts vary depending upon various factors. Here, we studied the antimicrobial effect of secondary metabolites of *Bituminaria* sp. genotypes and explored whether this effect changes with different growing stages of the plant. The results show that extracts obtained from 12 *B. bituminosa* genotypes were not significantly effective on Gram-negative bacteria but highly effective on Gram-positive bacteria and eukaryotic yeast. Of these genotypes, 9 and 12 genotype were more effective than the rest. Observations suggest that the extracts from the plants are more effective during the beginning of flowering stage than the growth and budding stages for the bacteria studied. It could be due to the differences in the nature of the metabolites and their quantity.

Keywords: Arabian pea, Brewer's Yeast, Pitch trefoil

Many plant species contain secondary metabolites. These secondary metabolites not only give a distinctive odour to the plant but also play a role in the defense mechanism of the plant, and have beneficial effects on human physiology and diseases due to their bioactive molecules[1-8]. These substances are used in the cosmetic industry for the treatment of vitiligo, psoriasis, fungus, eczema, sunburn, cancers, as well as mediterranean anemia, inflammation desiccant, sedative and allergen diseases[9,10]. Secondary metabolites which have pharmacological effect in *Bituminaria bituminosa var bituminosa* can be counted as furanocoumarins, isoflavonoids and pterocarbans[11].

The secondary metabolites that production in plants have a great diversity in biological activity. These compounds are in fact part of the defense mechanisms of the plants. Secondary metabolites released into the environment directly or indirectly, positive or negative effects on the plants. This effect, known as allelopathy, affects the germination and plant growth of the crop following the release of plant parts containing secondary metabolites into the soil[12].

Secondary metabolites of plants are also effective on microorganisms[3]. These metabolites are also important in terms of protection of humans from infections, to reduce the loss of yield from plant-pathogens[13]. The secondary metabolites in the leaf have a lethal or inhibitory effect on the micro-organisms in the soil. Although it is a disadvantage for conversion of organic substances to inorganic substances, it is important in terms of prevention of diseases in the plant caused by the microorganisms[14].

The secondary metabolite content of the plants can vary from species to species or between variation within the species. In addition, environmental factors may affect the amount of these metabolites. In this study, we explored how the secondary metabolite contents of plants change during its growth stages including flowering and budding, and also how it affects microorganisms. In addition, we have demonstrated the variation between different *Bituminaria bituminosa var bituminosa* genotypes grown in the same environment.

Materials and Methods

Identification and cultivation of plants

Bituminaria bituminosa (L.) C.H.Stirt. seeds were collected from 3 provinces and 11 different locations: Samsun (Kozağzı, Çarşamba, Bağkur, Baruthane, Nebyan, Kurupelit and Kavak), Sinop (Kanlıçay and

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Tıngıroğlu) and Kastamonu (Çatalzeytin, İnebolu) in 2012. Besides, one genotype of Spain origin was also used in the study (Table 1 and Fig. 1).

Plants were diagnosed at the flowering time by one of the authors, Prof. Zeki Acar, an expert on the breeding and genetics of the *B. bituminosa* plant. Seeds collected from the above locations were cleaned after identification and dried at 30°C. Then seeds were scarified using sandpaper and sown into seed trays. Seedlings were transplanted to Experimental Field in Samsun with 70×70 cm spaces in autumn during 2016. Twelve different genotypes collected from different localities are grown in the same area and under the same conditions. Experimental area soil properties were loamy with pH of 6.45 and 1.38% organic matter at the depth of 30 cm. The plants were harvested at the beginning of growth, budding, and in the beginning flowering, and the leaves were separated. Then the leaf samples were dried under natural conditions and then extracted with a Soxhlet apparatus[15].

Preparation of extracts of plant samples

About five grams of dried leaf samples were taken, extracted in 150 mL of 96% ethyl alcohol for 10 h in a Soxhlet apparatus, and the ethyl alcohol was removed in the evaporator[15]. Before antimicrobial activity study, the dry substances of all samples were weighed and stock solution was prepared in equal concentrations.

Antimicrobial activity of extracts of plant samples

Antimicrobial activity of extracts obtained from plants was tested on 5 microorganisms according to CLSI standards for antimicrobial susceptibility testing (CLSI 2020)[15]. For MIC tests, *Escherichia coli* W3110, *Bacillus cereus* 702 ROMA, *Salmonella enterica serovar typhimurium* ATCC 14028, *Staphylococcus aureus* ATCC 25923, *Saccharomyces cerevisiae* ATCC 9763 were used. Each of the microorganisms was seeded in 5 mL nutrient broth medium and pre-cultures were prepared for 18 h at 37°C at 160 rpm. After incubation, the density of the bacteria was adjusted to 0.1 absorbance at OD_{600} nm and then 400 µL of bacterial culture was added to 60 mL of sterile nutrient broth. From this culture, 100 µL of culture in the first well and 100 µL of culture were added to the 96-well microplate. About 100 µL of plant extracts were added to the first well containing 100 µL of bacterial culture and ½ serial dilution was performed. Plates were incubated at 37°C for 18 h and MIC values were determined the next day. Experiments were repeated by narrowing the interval at close concentrations of the MIC value determined in order to determine the most accurate and clear value. To the 96-well microplate, 120 µL in the first well, 100 µL in the other wells, 140 µL in the first well and 100 µL in the other wells, respectively. About 100 µL of plant extracts were taken from the first well containing bacterial culture and serial dilution was performed. Ethyl alcohol used as solvent was also tested as a control group[15]. In MIC experiments, one drop was taken from the wells without growth and allowed to grow on Nutrient Agar media. Therefore, it was determined whether the inhibition was caused by a static or cidal effect. Thus, cidal concentration values were determined.

Antimicrobial effect was also evaluated using disc diffusion method. For this purpose, microorganism cultures were prepared on nutrient broth medium at 37°C for 18 h. The absorbance of this culture was adjusted to 0.1. After this, 0.1 mL of culture was taken and spread on dried nutrient agar medium. Sterile 6 mm discs were placed on the Petri dish. The discs were loaded with 10, 20 and 30 µL of extracts and the zone diameters formed at the end of incubation at 37°C for 24 h were recorded. In

Table 1 — Localities of *Bituminaria bituminosa* genotypes

No	Collection Location	Localities	
		North	East
1	Spain	-	-
2	Kastamonu İnebolu	41° 58' 32.8"	33° 46' 10.4'
3	Kastamonu-Çatalzeytin	41° 57' 48.4"	34° 09' 07.8'
4	Sinop Kanlıçay	41° 40' 40.3"	35° 22' 22.8'
5	Samsun-Kozağzı	41° 28' 05.1"	35° 49' 56.8'
6	Samsun-Çarşamba	41° 04' 35.1"	36° 40' 09.0'
7	Samsun-Bağkur	41° 18' 39.0"	36° 20' 02.5'
8	Samsun- Baruthane	41° 19' 08.5"	36° 19' 13.6'
9	Samsun-Nebyan	41° 23' 35.9"	35° 59' 06.2'
10	Samsun-Kurupelit	41° 22' 16.0"	36° 11' 46.7'
11	Sinop-Tıngıroğlu	41° 47' 41.0"	35° 00' 23.0"
12	Samsun-Kavak	41° 03' 14.4"	35° 56' 59.8"

Fig. 1 — Localities of twelve *Bituminaria bituminosa* genotypes from 3 provinces of Turkey on Google Earth map

addition, ready-made discs of commercially available kanamycin (20 µg/mL) and nystatin (20 µg/mL) antibiotics were used as controls. The studies were performed with four independent replications and averaged.

Results and Discussion

B. bituminosa was taken from 12 different geographical points, and were grown in the same environment (Samsun). The extracts of the leaves taken from the beginning of growth (G stage), beginning of flowering (F stage) and budding stage (B) of *B. bituminosa* were investigated for antimicrobial effects on the identified microorganisms. Two Gram-negative (*E. coli* and *S. typhimurium*), two Gram-positive (*B. cereus* and *S. aureus*) and one eukaryotic (*S. cerevisiae*) microorganism were used. The MIC values were first determined and then the cidal values were determined to show whether this inhibition was caused by death or growth inhibition. In addition, disc diffusion method was used too. Thus, our objective was to compare the effects on microorganisms of leaf extracts from different development stages of different genotypes of the same species collected from different geographic points, then grown in the same environment.

In this study, it was found that the leaf extracts of 12 genotypes obtained during the growth start stage (G) had no effect on *S. typhimurium*. As the amount of ethanol used and the extract concentration that affects were the same as the concentration which inhibited to *S. typhimurium* and other microorganisms, it was obvious that the effect was caused by ethanol. Therefore, concentrations below 1012.5 µg/mL were considered as effective on microorganisms. The degree of effectiveness was compared between the values obtained from extracts. Only genotype 6 was found to be effective on *S. typhimurium* at a concentration of 675 µg/mL. It was also found that leaf extracts taken from the beginning of growth stage of genotypes 2, 3, 4, 7, 8, 9, 10, 11 and 12 had no effect in Gram-negative *E. coli*. The genotypes 1, 5 and 6 were found to have some effect on *E. coli* (506 µg/mL) (Table 2-G stage). It was determined that the extracts obtained during the G stage were highly effective on Gram-positives, especially *B. cereus* (41-165 µg/mL). Comparing the efficacy of leaf extracts obtained during growth stage (G); the extracts of genotypes 2 (41 µg/mL) and 3 (41 µg/mL) on *B. cereus* were the most effective, whereas genotype 4 was the least effective genotype at 165 µg/mL. Genotype 12 (1012.5 µg/mL) had no effect on *S. aureus*, and genotype 6 (210 µg/mL) and 3 (253 µg/mL) were found to be the most effective genotypes. It was observed that the extract at the beginning of growth of genotype 3 had an effect on both *S. aureus* and *B. cereus*. It has also been found to be effective (range 52-165 µg/mL) on *S. cerevisiae*, which is a eukaryotic organism. Genotype 1, 2 and 5 (52 µg/mL) were found to be the most effective genotypes, but genotype 7 was 165 µg/mL on *S. cerevisiae*.

Budding stage (B) in Table 2 shows that the most effective genotype on *B. cereus* is genotype 9 and 12, while the least effective genotypes are genotypes 7, 10 and 11. Similarly, genotype 9 (82.5 µg/mL) was the most effective genotype on *S. aureus* and genotypes 1, 2, 4, 10 and 11 (1012.5 µg/mL) were not effective. The genotype 9 was the most effective genotype for both the bacteria, and genotypes 10 and 11 were the least effective genotypes. Genotypes 7-12 were more effective on *E. coli*, while the other genotype 1-6 did not show any effect. On *S. typhimurium*, only genotype 1 showed effect (506 µg/mL)

From Table 2, the efficacy of the extracts obtained from 12 genotypes during the beginning of flowering (F code samples) can be seen that no genotype has any effect on *S. typhimurium*. When the results for *E. coli* were evaluated, it was determined that all genotypes except for genotype 7 during flowering stage (F) were found to be effective on *E. coli*. Our studies with Gram-positive bacteria show that *B. bituminosa* genotypes at flowering stage are particularly effective on Gram-positive bacteria. It was determined that genotype 9 and 12 were the most effective genotypes on *S. aureus* among the extracts obtained during flowering stage, and genotype 12 and 9 were the most effective genotypes on *B. cereus* (10.3 µg/mL) and *S. cerevisiae* (26 and 20.6 µg/mL, respectively).

While the effect of extracts obtained from genotype 7, 8, 9, 10, 11 and 12 on *E. coli* was higher during the budding stage (B), it is seen that these genotypes except 8 are most effective on *B. cereus*, *S. aureus* and *S. cerevisiae* during the flowering stage (F). The extracts of flowering stage from genotype 4, 5, 6 were more effective on *B. cereus* and *S. aureus*, but growing stage extracts of genotypes 5 and 6 was more effective on *E. coli* than other stages.

Table 2 — Antimicrobial activity of leaves extracts obtained during early growth stage (G), budding stage (B), and flowering stage (F). Minimal ınhibition concentration (MIC, µg/mL), Minimal cidal concentration (MCC, µg/mL)

		Gram-negative bacteria				Gram-positive bacteria				Eukaryote	
		Escherichia coli		*Salmonella enterica serovar typhimurium*		*Bacillus cereus*		*Staphylococcus aureus*		*Saccharomyces cerevisiae*	
Genotype	Stage	MIC	MCC	MIC	MCC	MIC	MCC	MIC	MCC	MIC	MCC
	G1	506	2025	1012.5	2025	63	63	506	675	52	63
1	B1	1012.5	2700	506	4050	210	210	1012.5	2025	165	210
	F1	506	2025	1012.5	2025	330	420	1012,5	1012.5	253	253
	G2	1012.5	2700	1012.5	4050	41	52	675	675	52	82.5
2	B2	1012.5	2700	1350	2700	210	210	1350	1680	165	253
	F2	506	2025	1012.5	2025	210	210	506	1012.5	210	210
	G3	2025	3370	1012.5	4050	41	41	253	506	63	63
3	B3	1350	3370	1350	2700	52	52	420	675	105	105
	F3	840	3370	1012.5	2700	26	26	420	420	105	105
	G4	1012.5	3370	1012.5	3370	165	165	840	1350	126	165
4	B4	1012.5	2700	1012.5	3370	82.5	82.5	1012.5	1012.5	165	165
	F4	506	4050	1012.5	2025	20.6	20.6	210	330	105	105
	G5	506	2025	1012.5	2700	52	52	506	506	52	82.5
5	B5	1012.5	2700	1012.5	2700	52	52	506	506	165	165
	F5	506	2025	1012.5	2025	26	26	165	420	105	105
	G6	506	2025	675	2025	82,5	82.5	210	253	63	63
6	B6	1350	3370	1350	3370	26	26	330	330	82.5	82.5
	F6	840	3370	1350	2700	20.6	20.6	210	506	52	52
	G7	1012.5	4050	1012.5	2025	105	105	506	1012.5	165	165
7	B7	506	2700	1012.5	3370	420	675	675	1680	210	210
	F7	1012.5	2025	1012.5	2025	26	26	506	1012.5	82.5	82.5
	G8	1012.5	2700	1012.5	2700	105	105	420	840	105	105
8	B8	506	2700	1012.5	2700	105	253	506	1350	105	105
	F8	840	2025	1012.5	2025	210	210	506	1012.5	165	165
	G9	1012.5	2700	1012.5	2025	82,5	125	675	1012.5	82,5	82.5
9	B9	675	2700	1350	3370	20.6	20.6	82.5	82.5	52	52
	F9	840	3370	1012.5	2700	10.3	10.3	52	82.5	20.6	20.6
	G10	1012.5	3370	1012.5	2700	52	82.5	420	675	82,5	82.5
10	B10	506	2700	1012.5	2025	420	675	1012.5	2025	253	253
	F10	840	2700	1350	2025	20.6	20.6	253	420	52	52
	G11	1012.5	4050	1012.5	3370	82,5	105	506	675	105	105
11	B11	506	2700	1012.5	2025	420	675	1012.5	2025	253	253
	F11	840	2700	1012.5	2025	26	26	506	675	82.5	82.5
	G12	1012.5	4050	1012.5	3370	82,5	105	1012.5	1350	126	165
12	B12	675	3370	1012.5	2700	20.6	26	165	330	82.5	82.5
	F12	675	3370	1012.5	2700	10.3	10.3	41	52	26	26

When the results are evaluated together, it is seen that genotype 9 and 12 are effective genotypes on Gram-positive bacteria and *S. cerevisiae* which is an eukaryotic organism. It suggests that the active substance is produced more during the beginning of flowering stage than other stages. Furthermore, when the cidal and MIC values are compared, it can be found that while the active substances have a static effect on Gram-negative bacteria, but a cidal effect on the Gram-positive and yeast at MIC value. In the most effective genotypes 9 and 12, the effect increased from growth to beginning of flowering. In this result, it can be stated that especially in fight with Gram-positive and eukaryotic pathogens, leaf extracts of genotypes 9 and 12 in beginning of flowering stage will be more effective.

Antimicrobial activity of genotypes 9 and 12, were found to be the most effective according to MIC values (Table 2) which was also demonstrated by disc diffusion (Fig. 2 and Suppl. Table 1. *All supplementary data are available only online along with the respective paper at NOPR repository at http://nopr.res.in*). Transparent zone diameters formed by the effect of extracts can be seen in Fig. 3. Overall results obtained by the disc diffusion method for the all the 12 genotypes at three different stages (Suppl. Table 1) substantiated the observations made

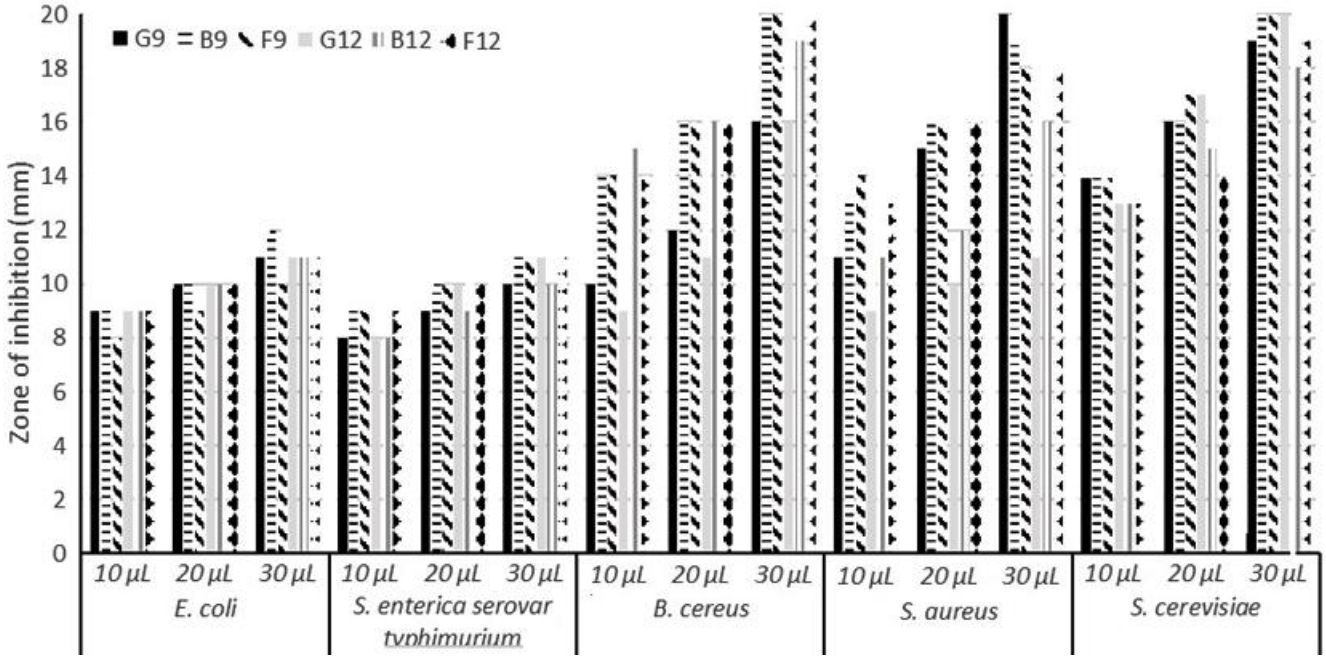

Fig. 2 — Disc diffusion results showing antimicrobial activity of genotypes 9 and 12 for the early growth (G), budding (B), and flowering (F) stages

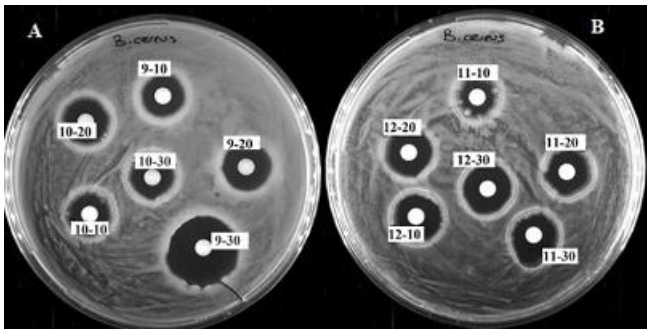

Fig. 3 — Zone of Inhibition displayed by of leaf extracts of *Bituminaria bituminosa* genotypes 9 and 12 at flowering stage on *B. cereus*. (A) Loading 10, 20 and 30 µL of genotypes 9 and 10 on discs; and (B) Loading 10, 20 and 30 µL of genotypes 11 and 12 on discs

in the MIC test (Table 2). According to the disc diffusion results, leaf extracts of *B. bituminosa* affected the Gram-negative bacteria at least, while extracts were most effective on *B. cereus* and *S. cerevisiae*. Fig. 3 shows the highest zone diameter (20 mm) on *S. cerevisiae* detected for extracts of B9, F9 and G12 (in 30 µL). Genotypes 9 and 12 have been found most effective on *S. cerevisiae* and zone diameters were over 18 in 3 different periods. While genotypes 1, 2, 3, 4, 5 and 6 were generally more effective during the growth phase, 7, 8, 9, 10, 11, 12 genotypes showed effective inhibition at the flowering stage on Gram-positive bactieria and yeast (Figs 2 & 3 and Suppl. Table 1). According to disk diffusion results, application of 10 µL on Gram-negative bacteria in all stages of all genotypes had no effect because it is the same as the solvent. The zone diameters of *E. coli* and *S. typhimurium* were approximately 11 mm in different stage of 9 and 12 (30 µL in Fig. 3).

The active substances on microorganisms in plants are generally grouped into five groups: phenolics, terpenoids essential oils, alkaloids, lectins polypeptides and polyacetylenes[16-20]. As many as 12000 of such secondary metabolites have been reported to be isolated until now, accounting for only 10% of all aromatic compounds[21,22]. Changes in the structure of these compounds such as coumarins also create differences in their antimicrobial activity[23,24]. Phenolic compounds are divided into two groups as phenolic acids and flavonoids. Phenolic acids; hydroxy benzoic and hydroxy cinnamic acids are divided into two groups, while flavonoids; anthocyanidins, flavones, flavonols, flavanones, catechins, Isoflavones and leucoanthocyanidins are divided into six groups as proanthocyanidins[25,26].

Earlier studies on *B. bituminosa* have reported high concentrations of furanocoumarins (psoralen and angelicin), pterocarpans (Erybraedin C, bitucarpin A and B) and flavonoids (daidzin and isoorientin)[11,27].

Plants usually accumulate furanocoumarins in fruits. Furanocoumarins accumulation in leaves has been shown to vary seasonally in summer, winter and spring months. Irrigation to reduce water stress in semi-arid soils has been found to increase the concentration of fruit psoralen and leaves do not change much[28,29]. Pecetti *et al*.[30] has demonstrated secondary metabolite differences in the leaves of *B. bituminosa* in summer and winter in Italy.

In the study conducted by Kaçar[31] investigated the effect of leaf extracts of *Psaroela bituminosa* on *E. coli*, *E. faecium*, *S. aureus*, *S. epidermidis* and *B. subtilis* and found it to be effective on *B. subtilis*. *E. faecium* used in the study was Gram-positive and the activity on this bacterium was low. It shows that the effect was also selective among Gram-positive ones. Similar results were obtained on *B. cereus* and *Staphylococcus aureus* in spite of solvent differences, i.e., ethyl alcohol instead of DMSO as solvent. Bammou *et al*.[32] who studied antimicrobial activities in water extracts of leaves and flowers of *B. bituminosa* showed that the leaves to be more effective than flowers, and the highest effect was found on *Bacillus subtilis* (13.33±0.57 mm). In our study too, the largest effect of different stages of *B. bituminosa* were on *B. cereus*.

Among the methylene chloride, ethyl acetate and butanol leaf extracts of *B. bituminosa* the methylene chloride extract was most effective on *S. aureus* among 3 wild type and 7 clinical isolates studied[13]. Bacillus group was not used in this study. However, according to the results, no generalization could be made in terms of the effectiveness of the extracts obtained from other solvents on Gram-negative or positive. According to the results of our study, *B. bituminosa* is clearly effective on Gram-positives. Ethyl alcohol was used as solvent in our study. The solvent difference is a significant difference. In addition, the differences in the conditions in which the plants are produced and grown may vary the type and amount of secondary metabolites.

In our study, we observed difference in the antimicrobial activities of the leaves obtained at different growth stages indicating different amounts of secondary metabolites present during the development stages of the plant. Similarly, differences between genotypes also indicate difference in the amount of secondary metabolites in different genotypes.

Studies report presence of psoralen and angelicin (furanocoumarins) in the leaves and stems of *B. bituminosa*[33], and also during its different growth stages and season[34]. Among the samples taken in April, August and November, the August samples showed the highest values[35]. Increased temperature and UV light intensity also resulted in increased furanocoumarin content[36]. As the the quantity of these contents changes during various growing stages as well as different parts of the plant, its antimicrobial activity also vary accordingly.

In the study where the above-ground parts of *B. morisiana* and *B. bituminosa* plants were examined, the isoflavone 8-prenyldaidzein, was isolated only from *B. morisiana*[27]. In a chemotaxonomic study on the roots of *Psoralea corylifolia* grown in Africa, presence of daidzein was detected though no substance isolation was done[37]. Works on production of daidzein through *Psorelea* tissue culture revealed both intra- and inter-specific variations[38]. Studies on isoflavone production in *P. corylifolia* by sprouting showed that root-derived callus cultures produced maximum amount of daidzein, and genistein by leaf-derived callus[39]. We have also analyzed the amount of psoralen, angelicin, genistein, daidzein and daidzin substances in *B. bituminosa* extracts by LC/LCMS (Data not provided). When these results were evaluated, the antimicrobial effect could not be correlated with the different stages of these substances obtained from the plant and the amounts of the substances.

Plants can synthesize different metabolic substances in their developmental processes. The flowering-fruit production stage and the growth stage of plants are stages to have quite different dynamics. Therefore, changes in the production of secondary metabolites according to the stages require determination of the acquisition times of these metabolites. Our results in the current study suggest that active chemicals for antimicrobial in *B. bituminoria* (especially genotype 9 and 12) on Gram-positive and eukaryote are more common in the flowering stage. The extracts obtained from *B. bituminosa* genotype 12 were not effective on Gram-negative but highly effective against Gram-positive bacteria and eukaryotic yeast. Of these genotypes, 9 and 12 proved more effective than the rest. Further, the secondary metabolites of *B. bituminosa* are found more effective on the bacteria during beginning of flowering stage, and this could be due to the difference in the contents,

both in terms of quality and quantity. Effect of the extracts of *B. bituminosa* on *B. cereus* though shows its potential for use against pathogens of Bacillaceae it requires further detail investigation.

Conclusion

The antimicrobial activity of the leaf extracts of *Bituminaria bituminosa* sampled during different growth stages (early growth, budding and flowering) showed varied response when tested against Gram-negative (*Escherichia coli* and *Salmonella enterica serovar typhimurium*) and Gram-positive (*Bacillus cereus* and *Staphylococcus aureus*) bacteria and eukaryote (*Saccharomyces cerevisiae*). The *B. bituminosa* leaf extract was more effective in the flowering stage than the other two stages, particularly against Gram-positive bacteria (especially *B. cereus*). The effect of the antimicrobial activity exhibited a difference between its genotypes. Over all, the results suggest that the antimicrobial activity of the leaf extract of *B. bituminosa* vary with its different growth stages and also with the genotype. Among the 12 genotypes of *B. bituminosa var bituminosa* collected from three provinces Samsun, Sinop and Kastamonu of Turkey, only the flowering stage leaf extract of two genotypes showed potential activity, particularly against Gram-positive bacteria *B. cereus*. This difference could be attributed to different secondary metabolites present in the plant which may vary during its growth stages and also between their genotypes.

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Conflict of interest

Authors declare no conflict of interests.

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