

## ANTIBACTERIAL AND ANTIBIOFILM PROPERTIES OF *PHLOMIS* AND *STACHYS* SPECIES

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**Keywords:** Antibacterial activity, Antibiofilm activity, *Phlomis*, *Stachys*

### Abstract

The ethyl acetate and methanol extracts of *Phlomis pungens* var. *pungens*, *P. nissolii*, *P. armeniaca* and *Stachys byzantina*, *S. cretica* L. subsp. *mersiaea* and *S. cretica* L. subsp. *Smyrnaea* were evaluated for their antibacterial and antibiofilm activities. Five bacterial species were used are *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Shigella sonnei*, *Salmonella typhimurium* and *Yersinia enterocolitica*. The ethyl acetate and methanol extracts of plant species showed good antibacterial activity against all bacterial strains. More significantly, ethylacetate and methanol extracts of all *Phlomis* species were found to be more effective on degradation of mature biofilm against all used bacterial strains than extracts of *Stachys* species. The findings of the present study highlight the promising role of *Phlomis* and *Stachys* extracts as new lead structures in the search for novel antibacterial and antibiofilm agents.

### Introduction

Although traditionally regarded as environmental phenomena, bacterial biofilms are increasingly considered to play a role in many human infections (Hung and Henderson 2009). Biofilm included living bacteria are intrinsically nonreactive to the host immune response and are thousand times more resistant to antibiotics (Caraher *et al.* 2007). Becoming increasingly resistant to bacteria, it led to conventional antibiotic therapy inactive. Eventually, researchers have undertaken to explore alternative therapeutical agents including phage therapy (Sulakvelidze *et al.* 2003, Fischetti 2005) and human antimicrobial peptides (De Semet and Contreras 2005).

The whole components of the *Phlomis* are used in folk medicine containing flowers (*P. aspera*, *P. rotata*), leaves (*P. aspera*, *P. cephalotes*), seeds (*P. cephalotes*) and roots (*P. nepetaefolia*). *Phlomis* is a great beneficial group to the finding and usage of natural curative products. According to literature, a wide range of *Phlomis* species throughout the world have the same manner of use qua herbal tea to heal several disorders such as diabetes, gastric ulcer, hemorrhoids, inflammation, and wounds. The antimicrobial, antidiabetic, the antinociceptive, antiulcerogenic, anti-inflammatory, antiallergic and antioxidant effects of some *Phlomis* species extracts were also reported (Demirci *et al.* 2009, Ozkan *et al.* 2009). During the recent years, there has been a growing interest in the information available on the compositions and pharmacological properties of new substances recognized from *Phlomis* species.

*Stachys* plants have been used in traditionally folk medicine as astringent, wound-healing, anti-diarrheal, anti-nephritic and anti-inflammatory agents in many countries over the years. Moreover, antimicrobial, antioxidant and cytotoxic activities of some *Stachys* species are determined (Bhattacharjee *et al.* 1982, Saeedi *et al.* 2008, Dulger and Aki 2009). According to previous studies, the extracts of *Stachys* species exhibited concentration dependent antibacterial activity against *K. pneumoniae*, *S. aureus*, *E. coli*, *P. aeruginosa*, *S. sanguis* (Saeedi *et al.* 2008, Dulger and Aki 2009). Pharmacological surveys have showed that extracts or compounds of *Stachys* species has propound notable antibacterial, antifungal, antioxidant, anti-inflammatory and

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hypotensive activities (Piozzi 2011). Attributable to their diverse significant effects, future investigations should be find out on the pharmacological advance of *Stachys* extracts and their contents.

Recent findings showed that natural phenolic and aromatic substances which are isolated from medicinal herbs have an antibacterial and anti-biofouling effect on biofilm formation (Jagani *et al.* 2009). Previously antibiofilm and antimicrobial activity of various plant extracts were reported (Quave and Smeltzer 2009). In the available literature there is not much data about the antibiofilm activity of *Phlomis pungens* var. *pungens*, *P. nissolii*, *P. armeniaca* and *Stachys byzantina*, *S. cretica* L. subsp. *mersiaea*, *S. cretica* L. subsp. *smyrnaea*. Thus in the present study, antibacterial and antibiofilm activities of the ethyl acetate and methanol extracts of two plant species, *Phlomis* and *Stachys* which have been used as medicinal plants over the many years were investigated.

### Materials and Methods

*Phlomis armeniaca*, *P. nissolii* and *P. pungens* var. *pungens* were collected from Kemer kaya town, Bolvadin-Afyonkarahisar-Turkey on June, 2013, Cumra-Bozkır highway, Konya-Turkey on July, 2013 and Karagöl-Cubuk, Ankara-Turkey on July, 2013, respectively. *Stachys byzantina*, *S. cretica* L. subsp. *mersiaea* and *S. cretica* L. subsp. *smyrnaea* were also collected from Kemer kaya town, Bolvadin-Afyonkarahisar-Turkey on June, 2013, Keçiborlu-Aydoğmuş highway, Isparta-Turkey on June, 2014 and Isparta-Bucak-Turkey on June, 2014, respectively. All plants were obtained by Dr. Basak Gokce, Department of Pharmacy, Suleyman Demirel University.

The voucher specimen was deposited at the Herbarium in Mugla Sıtkı Kocman University, Mugla-Turkey. Taxonomic identification was confirmed by the senior taxonomist Dr. Olcay Ceylan, in Department of Biology, Mugla Sıtkı Kocman University.

Air-dried samples (30 g) of the aerial parts (leaves) of plant species were extracted by using a Soxhlet extractor for 5 hrs with 250 ml of ethyl acetate and methanol. Ethyl acetate and methanol were then removed by rotary evaporator. All extracts were stored at 4°C until using for assays. Ethyl acetate and methanol extracts were dissolved in 20% of dimethyl sulfoxide (DMSO, Carlo-Erba, Milan, Italy), and sterile filtered (0.2 mm; nylon filter) and stored in sterile vials at -20°C prior to use for antibacterial and antibiofilm assays. All experiments were carried out Department of Biology, Bursa Uudag University.

The following bacteria were used: *Bacillus subtilis* ATCC6633, *Pseudomonas aeruginosa* ATCC35032, *Shigella sonnei* ATCC25931, *Salmonella typhimurium* ATCC14028 and *Yersinia enterocolitica* ATCC9610. Bacterial species were obtained from the Department of Microbiology, Faculty of Medicine, Uludag University. For antibacterial assay, a single colony in Luria- Bertani (LB) agar plates of all strains was transferred to Muller Hinton broth (MHB) and then incubated at 37°C for 24 hrs. After incubation it was adjusted to a concentration of  $1 \times 10^6$  colony forming units per ml (CFU/ml) to add Muller Hinton agar (MHA). For antibiofilm assay, all the bacterial strains were cultured overnight at 37°C in LB medium.

Agar well diffusion assay was used to determine the antibacterial activity of plant extracts (Cheesbrough 2006). MHA plates that have been checked for sterility were seeded with 100 µl of an overnight broth culture of each bacterial isolate in sterile Petri dishes. The seeded plates were allowed to set after a uniform distribution of the bacterial isolate following slow rotation of the Petri dishes. A sterile stainless steel cork borer was used to cut 6 mm diameter of wells on the agar. The wells were filled with 100 µl of 5 mg/ml stock solutions of each extracts for *Phlomis* and *Stachys* species. One of the wells in each MHA plate was filled with 20% of DMSO as a control. The plates were then allowed to stand for 1 hr at room temperature to allow proper

diffusion of the extracts to occur. All the plates were incubated at 37°C for 24 hrs and observed for zones of inhibition. Diameters of zones were measured in millimeter (mm) and recorded.

The microplate method of Eloff (1988) was used to determine the MIC values for ethyl acetate and methanol extracts of *Phlomis* and *Stachys* species. Ten mg/ml stock solutions were prepared for each extract which was later transferred to 96-well microtiter plate (MTP) (Sigma-Aldrich, St. Louis, MO, USA) in 100 µl aliquots. Prepared bacterial suspensions were pipetted into each well in an equal volume. Media containing bacterial suspensions – 20% of DMSO were served as positive growth control. The microplates were incubated at 37°C for 24 hrs and absorbance values at 600 nm were read spectrophotometrically. MICs were recorded as the lowest concentrations of extracts. Experiments were performed in triplicate.

The effect of 2 × MIC of *Phlomis* and *Stachys* ethyl acetate and methanol extracts on the biofilm formation was determined by quantifying the biofilm biomass through microtiter plate (MTP) assay (Nithya *et al.* 2010). Briefly, to test effects of plant extracts on biofilm formation, 100 µl of 2 × MIC of *Phlomis* and *Stachys* extracts were added to wells prior to bacteria inoculation. Overnight cultures of bacterial strains were adjusted to 1 × 10<sup>6</sup> CFU/ml and then 100 µl of inocula were added into MTP contained medium. MTPs were incubated in 37°C for 24 hrs. After incubation, MTPs were emptied by removing the media along with free-floating planktonic cells and the wells were gently rinsed twice with sterile water. The surface-attached cells were stained with 0.1% crystal violet solution. After 15 min, crystal violet solution was discarded completely and wells were filled with 95% ethanol to solubilize crystal violet from the stained cells. The biofilm biomass was then quantified by measuring the absorbance at OD 590 nm using a UV-visible spectrophotometer. Experiment was repeated at three times and results were expressed average of three values.

## Results and Discussion

Antibacterial activities of ethyl acetate and methanol extracts of three *Phlomis* and *Stachys* species are illustrated in Table 1a, b. From the results it can be seen that these extracts were resulted in variable zones of inhibition (10 - 26 mm) for all bacterial strains used. DMSO as a control did not show antibacterial activity. In general, ethyl acetate and methanol extracts of six plant species have good antibacterial activity. Among bacterial strains, all bacteria were found to be more sensitive to ethyl acetate and methanol extracts of *P. nissolii* and *S. cretica* L. subsp. *smyrnaea* than other *Phlomis* and *Stachys* species. The largest inhibition zones were found in ethyl acetate extract of *P. nissolii* against *P. aeruginosa* (26 mm) and in methanol extract of *P. armeniaca* against *P. aeruginosa* (20 mm). In a previous study, the highest growth inhibition zones of *P. armeniaca* chloroform extract were observed against the medically important pathogens such as *S. aureus* ATCC 25923 and *S. aureus* Cowan 1 (32 mm), *L. monocytogenes* (29 mm), *B. cereus* (34 mm) and *B. subtilis* (33 mm). *P. pungens* var. *pungens* (9 mm for *C. albicans* and 8 mm for *C. tropicalis*) demonstrated anticandidal activity. But these plants did not show antibacterial effect on *P. aeruginosa* (Dogan *et al.* 2010). On the contrary of these results, we reported that ethyl acetate and methanol extracts of *P. armeniaca* and *P. pungens* var. *pungens* showed good antibacterial activity against not only *P. aeruginosa* (10 and 20 mm) but also *B. subtilis* (12 and 15 mm), *S. sonnei* (Not active and 16 mm), *S. typhimurium* (14 and 17 mm) and *Y. enterocolitica* (11 and 19 mm) (Table 1). In addition, ethyl acetate extract of *P. pungens* var. *pungens* was found more effective than methanol extract.

The two extracts of *Phlomis* and *Stachys* species used showing considerably antibacterial activity for each bacterial strains were selected to determine MICs. The MICs of extracts were determined ranging from 0.312 to 2.5 mg/ml (Table 1). On the contrary the present results MICs

of methanol extract of *P. pungens* were determined as 50 - 100 mg/ml against *K. pneumoniae* ATCC 4352, *P. aeruginosa* ATCC 27853, *B. subtilis* ATCC 6633, *S. aureus* ATCC 29213,

**Table 1. Antibacterial properties of ethylacetate (a) and methanol (b) extracts of *Phlomis* and *Stachys* species against tested bacteria.**

(a)						
Bacterial species	Ethyl acetate extracts of plants		MIC (mg/ml)		Zones of inhibition (mm)	
<i>B. subtilis</i>	1	a	0.625	1.25	18	12
	2	b	0.625	0.625	11	12
	3	c	<b>0.312</b>	0.625	16	19
<i>P. aeruginosa</i>	1	a	1.25	0.625	19	17
	2	b	1.25	<b>0.312</b>	11	10
	3	c	0.625	<b>0.312</b>	16	<b>26</b>
<i>S. sonnei</i>	1	a	<b>0.312</b>	<b>0.312</b>	16	10
	2	b	0.625	0.625	11	NA
	3	c	0.625	0.625	17	21
<i>S. typhimurium</i>	1	a	1.25	1.25	<b>25</b>	17
	2	b	1.25	1.25	13	14
	3	c	0.625	1.25	19	21
<i>Y. enterocolitica</i>	1	a	0.625	2.5	22	19
	2	b	1.25	2.5	17	11
	3	c	1.25	1.25	22	20
(b)						
Bacterial species	Methanol extracts of plants		MIC (mg/ml)		Zones of inhibition (mm)	
<i>B. subtilis</i>	1	a	1.25	0.625	17	12
	2	b	<b>0.625</b>	0.625	19	15
	3	c	1.25	1.25	21	14
<i>P. aeruginosa</i>	1	a	2.5	1.25	19	10
	2	b	1.25	0.625	15	<b>20</b>
	3	c	1.25	0.625	21	10
<i>S. sonnei</i>	1	a	<b>0.625</b>	<b>0.312</b>	20	NA
	2	b	<b>0.625</b>	<b>0.312</b>	19	16
	3	c	1.25	0.625	<b>27</b>	10
<i>S. typhimurium</i>	1	a	2.5	1.25	33	12
	2	b	1.25	1.25	17	17
	3	c	2.5	0.625	22	18
<i>Y. enterocolitica</i>	1	a	2.5	1.25	19	10
	2	b	1.25	1.25	17	19
	3	c	1.25	2.5	24	11

1 - *S. cretica* subsp. *smyrnaea*, 2 - *S. cretica* subsp. *mersiaea*, 3 - *S. byzantina*. a - *P. pungens* var. *pungens*, b - *P. armeniaca*, c - *P. nissolii*.

*S. epidermidis* ATCC 12228, *B. cereus* ATCC 11778, *S. enteritidis* KUEN 349, *P. mirabilis* CCM1944 and *E. coli* ATCC 25922 (Ozkan *et al.* 2009). In a previous study, methanol extracts of *Stachys byzantina*, *S. inflata*, *S. lavandulifolia* and *S. laxa* exhibited concentration dependent

antibacterial activity against *P. aeruginosa*, *E. coli*, *S. aureus*, *S. epidermidis* and *K. pneumoniae*. MICs of extracts were found to range from 10 - 40 mg/ml against all bacteria tested (Saeedi *et al.* 2008). In the present study, the results revealed that the ethyl acetate and methanol extracts of *Stachys* and *Phlomis* species with lower MICs (0.312 - 2.5 mg/ml) and higher inhibition zones (10 - 26 mm) against all bacteria tested have a strong potential as a source of antibacterial agents of nature origin.

Essential oils and plant extracts have been used in folk medicine since ancient times to cure several illnesses (Nazzaro *et al.* 2013). The most potent antibacterial substances against pathogenic microorganisms come from phenolic compounds such as carvacrol, eugenol and thymol (Hyldgaard *et al.* 2012). Phytochemical analyses of *Stachys* species have confirmed the occurrence of diterpenes, phenyl ethanoid glycosides, flavanoids and saponines (Khanavi *et al.* 2005). In such experiments, 3-O-octanoyl (+)-catechin has been shown to cause a reduction of 1000-folds or more in viable counts of EMRSA-16 (Stapleton *et al.* 2004). In a previous study, amounts of (+)-catechin as phenolic components of *Phlomis nissolii* and *P. pungens* var. *pungens* was importantly found high (Sarikurkcü *et al.* 2014). This would immediately appear to suggest that flavonoids such as (+)-catechin may be responsible for their antibacterial activity (Saeedi *et al.* 2008).

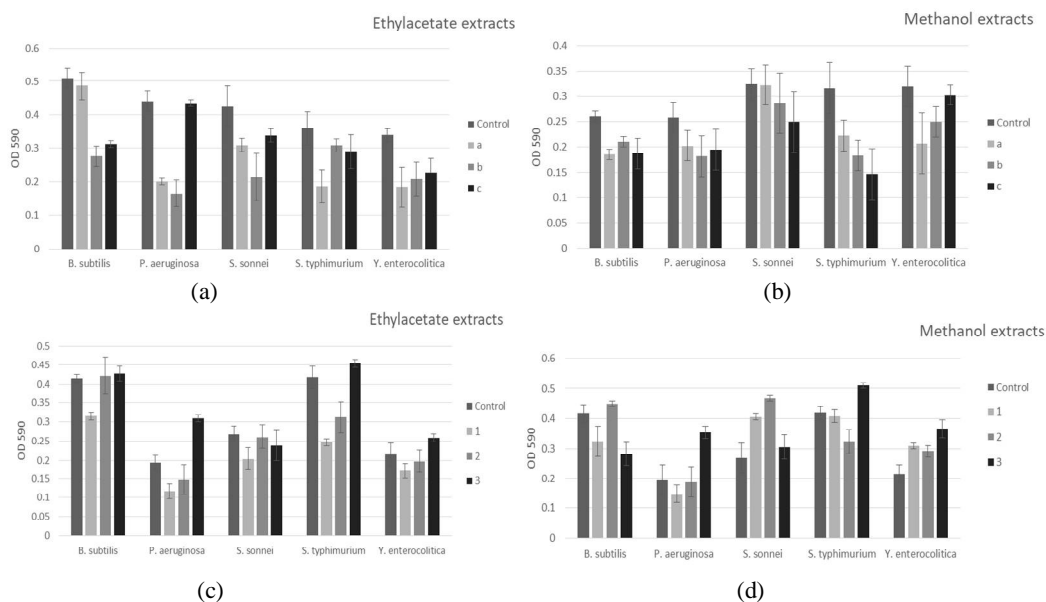


Fig. 1. Inhibitory effects of ethyl acetate (a, c) and methanol (b, d) extracts of *Phlomis* and *Stachys* species on mature biofilm, respectively. a - *P. pungens* var. *pungens*, b - *P. armeniaca*, c - *P. nissolii*, 1. *S. cretica* subsp. *smrymaea*, 2. *S. cretica* subsp. *mersiaea* and 3. *S. byzantina*.

Effects of *Phlomis* and *Stachys* extracts on mature biofilm were evaluated by crystal violet assay. Test bacterial biofilm assays showed that two extracts of all *Phlomis* species interfered degradation effect on only mature biofilms when compared with *Stachys* species, but not inhibited the starting phases of biofilm formation (datas not shown). *Stachys* species did not show considerable antibiofilm effect but the extracts of some *Stachys* species were found effective on degradation of mature biofilm (Fig. 1). In a previous study, the essential oil of *Phlomis* was found to be able to overcome the resistance of *S. epidermidis* at very low concentrations in both planktonic and biofilm form (Sarayreh *et al.* 2015). Sub-inhibitory concentrations (sub-MIC<sub>plank</sub>)

of *Phlomis brachydon* essential oil to polystyrene Petri dishes containing a suspension culture of the *E. coli* strain were also able to reduce the number of individual cells adhering to the polystyrene surface (Sarayreh *et al.* 2015). As distinct from bacterial strains in literature and as seen in Fig. 1, ethyl acetate and methanol extracts of three *Phlomis* species showed more considerable antibiofilm activity against some bacterial strains than *Stachys* species. As biofilm is a multifactorial phenomenon, *Phlomis* extracts could act on the evolution of mature biofilm.

Consequently, in the present study, plants extracts inhibited the growth of bacterial strains that cause some diseases and thus plants extracts may be antibacterial agents for controlling infections. *Phlomis* extracts also represent the first of many steps toward to development of new antibiofilm agents. Inhibition of biofilm by *Phlomis* extracts provides to control the effects of pathogenic bacteria without strong selection for drug resistance. The results indicate that these *Stachys* and *Phlomis* species used in traditional medicine have antibacterial and antibiofilm activities.

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(Manuscript received on 21 November, 2018; revised on 7 July, 2019)