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Evaluation of the stimulatory and inhibitory effects of *Malva sylvestris* leaf extract on some beneficial and pathogenic bacteria from the colon

Ahu Demirtaş

Department of Physiology, Faculty of Veterinary Medicine, Burdur Mehmet Akif Ersoy University, 15030, Burdur/TURKEY. Demirtaş A: ORCID: 0000-0003-2942-6243

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ABSTRACT

The aim of the present study was to evaluate the stimulatory and inhibitory effects of *Malva sylvestris* leaf extract on some selected beneficial and pathogenic bacteria from the colon to form a presupposition on its efficacy on intestinal health. The sensitivity of colon bacterial strains to *M. sylvestris* leaf extract was tested by a broth dilution method in the anaerobic cabinet. *Malva sylvestris* leaf extract stimulated the growth of *Bifidobacterium bifidum* from beneficial species starting from 0.06 mg/mL dose ($P<0.05$). The same stimulatory effect was observed for other beneficial species *Bifidobacterium infantis* and *Lactobacillus acidophilus* from 0.125 mg/mL dose ($P<0.05$) and that effect was more obvious for *B. infantis.* On the other hand, the extract did not have any effect on *Lactobacillus casei* up to 4 mg/mL dose. *Malva sylvestris* leaf extract also had a potential inhibitory activity against pathogenic *Escherichia coli*, *Clostridium perfringens*, and *Staphylococcus aureus* from 0.25, 2, and 4 mg/mL concentrations respectively ($P<0.05$). The dose of 8 mg/mL of the extract (MIC; minimal inhibitory concentration) completely inhibited *Fusobacterium nucleatum* ($P<0.05$), other enteropathogen, which is associated with colorectal cancer. It was concluded that *M. sylvestris* leaf extract at 0.06-8 mg/mL dose could have favorable effects on colon bacteria since the extract selectively promoted the most of the beneficial species' growth at this dose range while it had a potential inhibitory or inhibitory effect on pathogenic ones. Investigating the effects of *M. sylvestris* leaf extract on other colon bacteria and testing the *in vivo* effectiveness will contribute to a better understanding of its efficacy on colon microbiota and intestinal health.

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Introduction

Colon constitutes a complex community of bacteria, over 99% of which are anaerobic bacteria (Macfarlane and Macfarlane, 2003). Commensal or resident bacteria of the colon are considered as "beneficial" since they have remarkably crucial influences for the physiology, immunity, and susceptibility to diseases of the host (Kau et al., 2011). Consequently, disturbance of the colon microbiota can cause to expansion of pathogenic species and subsequent abnormal physiological states (Ahn et al., 1998). Recently, many studies have been focused on potential prebiotic and antibiotic effects of medicinal plants and plant metabolites on beneficial and pathogenic bacterial species from the colon microbiota (Phoem and Voravuthikunchai, 2012; Thapa et al., 2012; Goker and Demirtas, 2020).

Malva sylvestris, commonly known as mallow, is a plant native to Europe, Asia, and North Africa with dark green leaves and red to blue-purple flowers (Elsagh et al., 2015). This plant has a long history of

*Corresponding Author: Ahu Demirtaş
E-mail: ahu-demirtas@hotmail.com

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use in Mediterranean and European traditional medicine to treat inflammations, dermal infected wounds, bronchitis, and particularly digestive problems such as peptic ulcers, gastritis, enteritis, and colitis due to its high mucilage content and its colon cleansing properties (Gasparetto et al., 2012; Hamedi et al., 2015; Al-Rubaye et al., 2017). Aqueous extracts of aerial parts of *M. sylvestris* and also isolated polysaccharide of the plant were effective in preventing the inflammatory lesions of ulcerative colitis in the rats (Hamedi et al., 2015). The leaves have shown high effectiveness against swine constipation and in the treatment of mastitis in bovines when applied in enemas or compresses (Uncini-Manganelli et al., 2001). Phytochemical analyses of the *M. sylvestris* leaves have also relieved the presence of a wide range of phytochemical groups with antimicrobial properties such as flavonoids, alkaloids, phenolic acids, quinones, tannins, saponins, steroids, terpenoides, carotenoids, and unsaturated fatty acids (Barros et al., 2010; Dowek et al., 2020). There are reports about antibacterial activity of the extracts of *M. sylvestris* leaf on plant (Razavi et al., 2010), oral (Vahabi et al., 2019), and wound pathogens (Zare et al., 2012) and on some clinical isolates (Azadpour et al., 2016). However the literature is scarce regarding the effects of *M. sylvestris* leaf extract on colon bacteria especially on beneficial ones. Therefore, the aim of the present study was to evaluate the stimulatory and inhibitory effects of *M. sylvestris* leaf extract on some selected beneficial and pathogenic bacteria from the colon.

Materials and Methods

***Malva sylvestris* leaf extract:** The extract was provided by Kale Naturel Herbal Products Company, Ltd., Balikesir, Turkey. As specified by the manufacturer, *M. sylvestris* leaves were air dried, ground into 0 to 200 µm large particles and screened. Powdered plant leaves used for extraction in 80% ethanol (1/10, w/v) at 30°C for 4-5 h and then filtered. The extract was concentrated to 1/5 of its volume with a rotary vacuum evaporator at 35°C for 8 h. The drying process of the extract was performed using a laboratory-scale spray dryer. Afterwards, dry extracts liquefied in the mixer at an adequate ratio.

Microorganisms and growth conditions: The assay was carried out using the following bacterial species: *Bifidobacterium bifidum* ATCC 29521, *Bifidobacterium longum* subsp. *infantis* ATCC 15697, *Lactobacillus acidophilus* ATCC 4356, and *Lactobacillus casei* ATCC 393 as beneficial bacterial species. *Staphylococcus aureus* subsp. *aureus* ATCC 12600, *Escherichia coli* ATCC 11775, *Clostridium perfringens* ATCC 13124, and *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586 as pathogenic bacterial species. The cultivation medium was Mann Rogosa Sharpe (MRS) broth for *B. infantis, L. acidophilus*, and *L. casei*; MRS broth with 0.05% cysteine (MRS-C) for *B. bifidum*; Luria–Bertani (LB) medium for *E. coli*; tryptic soy broth (TSB) for *S. aureus*; and liquid form of medium 2 (Hobson, 1969) for *C. perfringens* and *F. nucleatum*. Medium 2 was prepared anaerobically according to Hobson (1969) with only slight modification. Trypticase peptone was used instead of casitone in medium 2 (Table 1). Ruminal fluid which was used as a component of the medium 2 brought from the slaughterhouse, mixed, and filtered through three layers of cheesecloth to partition into liquid and solid (digesta) fractions. The liquid fraction was centrifuged at 15000 rpm, and the clear supernatant was used as a component of the medium (Table 1). All strains were grown at 37°C for 24 h under an atmosphere of 80% N_2, 10% CO_2, and 10% H_2 in an anaerobic cabinet (Whitley DG250, Don Whitley, West Yorkshire, UK).

Table 1. Composition of medium 2 (for 100 mL)

Component	
Trypticase peptone (BD 211921 Bacto™)	1.0 g
Yeast extract (Sigma Y1625)	0.25 g
Mineral solution 1	15 mL
Mineral solution 2	15 mL
Clarified rumen fluid	20 mL
Resazurin (Sigma R7017)	0.0001 g
Sodium lactate (70% w/v)	1.0 g
Glucose	0.2 g
Maltose	0.2 g
Cellobiose (Sigma 22150)	0.2 g
Cysteine HCl (Sigma C7880)	0.05 g
$NaHCO_3$ (Sigma S5761)	0.4 g
Deionized water	to 100 mL

Mineral solution 1 - 3 g/L K_2HPO_4 (Sigma P3786); Mineral solution 2 - 3 g/L KH_2PO_4 (Sigma P9791), 6 g/L $(NH_4)_2SO_4$ (Sigma A4915), 6 g/L NaCl (Sigma S7653), 0.6 g/L $MgSO_4 \cdot 7H_2O$ (Sigma 230391), and 0.6 g/L $CaCl_2$ (Sigma C1016).

Sensitivity of bacterial species to *M. sylvestris* leaf extract: The sensitivity of colonic bacterial strains to *M. sylvestris* leaf extract was tested by a broth dilution method (CLSI, 2016) in the anaerobic cabinet. A stock solution was prepared by dissolving the plant extract in 50% ethanol. For broth microdilution, 20 µl of an overnight bacterial culture was transferred to wells of a 96-well plate (Flat bottom, Corning 3599) that

already containing 200 µl of two-fold serially diluted *M. sylvestris* leaf extract in the bacterial strain specific growth media. Final concentrations of extract were kept at the ranges of 0.015-8 mg/mL. Each strain was tested in triplicate wells. Plates were incubated for 24 h at 37°C in the anaerobic cabinet. Bacterial growth was detected with a microplate reader at 600 nm (Epoch, BioTek, USA). The minimal inhibitory concentration (MIC) was the lowest concentration of the extract that allowed no visible growth. A significantly lower OD600 value compared to control dose (0 mg/mL) was accepted as potential inhibitory activity (Ko et al., 2018) while significantly higher value was accepted as stimulatory effect (Das et al., 2015).

Statistical analyses: Statistical analysis was carried out by the use of one-way ANOVA followed by Dunnett's test. Each well of a 96-well plate was an experimental unit. A value of P<0.05 was taken to indicate a significant difference.

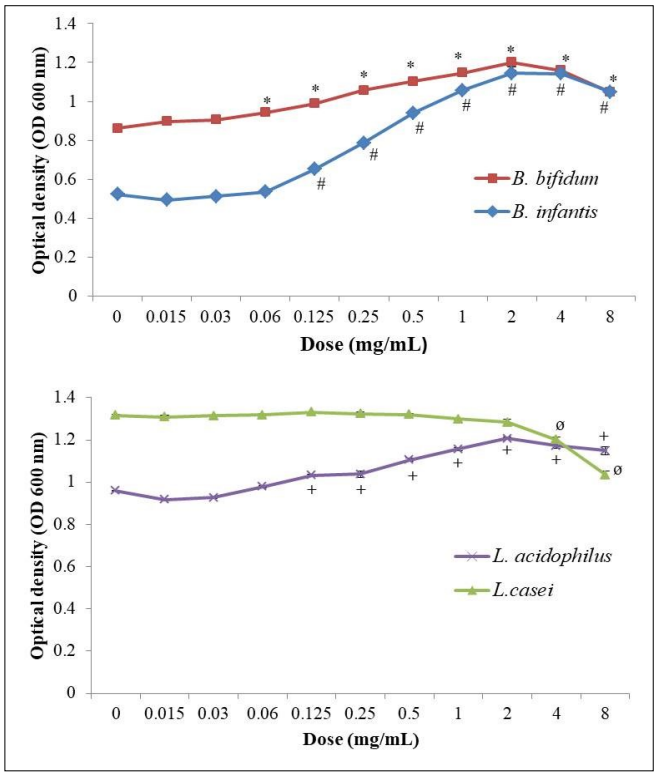

Figure 1. Effects of *M. sylvestris* leaf extract on beneficial colon bacteria. The results represent the mean ± standard error. *P<0.05, extract treated culture vs *B. bifidum* control; #P<0.05, extract treated culture vs *B. infantis* control; +P<0.05, extract treated culture vs *L. acidophilus* control; and øP<0.05, extract treated culture vs *L. casei* control. Control level was 0 mg/mL of the extract.

Results

Effects of *M. sylvestris* leaf extract on colon bacteria are presented in Figure 1 and Figure 2. *Malva sylvestris* leaf extract did not have inhibitory effect on

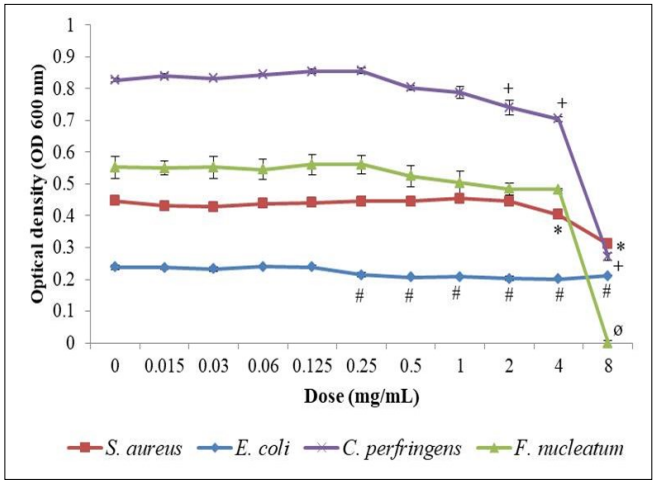

Figure 2. Effects of *M. sylvestris* leaf extract on pathogenic colon bacteria. The results represent the mean ± standard error. *P<0.05, extract treated culture vs *S. aureus* control; #P<0.05, extract treated culture vs *E. coli* control; +P<0.05, extract treated culture vs *C. perfringens* control; and øP<0.05, extract treated culture vs *F. nucleatum* control. Control level was 0 mg/mL of the extract.

bifidobacteria species and *L. acidophilus*. Besides, the extract stimulated the growth of *B. bifidum* starting from 0.06 mg/mL dose (P<0.05). The same stimulatory effect was observed for *B. infantis* and *L. acidophilus* from 0.125 mg/mL dose (P<0.05) and that effect was more obvious for *B. infantis*. The extract did not have any effect on *L. casei* up to 4 mg/mL dose, however showed a potential inhibitory activity from that dose (P<0.05). *Malva sylvestris* leaf extract also had a potential inhibitory activity against *E. coli*, *C. perfringens*, and *S. aureus* from 0.25, 2, and 4 mg/mL concentrations respectively (P<0.05). The extract, on the other hand, completely inhibited *F. nucleatum* at 8 mg/mL (MIC) (P<0.05).

Discussion

Many species of bacteria have adapted to grow in the colonic lumen with concentrations up to 10^{11} or 10^{12} cells/g of luminal contents (Guarner and Malagelada, 2003). Of these bacterial groups, the bifidobacteria is one of the most important genera in the human and animal intestinal tract with its role in the fermentation of the complex carbohydrates (Crociani et al., 1994), producing vitamins, enhancing immunity, and inhibiting invasion of potential pathogens (Shen et al., 2011).

Malva sylvestris leaf extract promoted the growth of *Bifidobacterium* species in a dose-dependent manner in the present study. The density of *B. infantis* nearly doubled in the high-dose groups. Same stimulatory effect was observed for *L. acidophilus* which is the other beneficial bacteria of the colon, which provides health-promoting effects (Shen et al.,

2011). *Malva sylvestris* is rich in polysaccharides such as mucilages, which are particularly responsible for the therapeutic effects of the plant in the gastrointestinal disorders (Gasparetto et al., 2011). It is reported that the mucilage content of *M. sylvestris* leaves is 17.2% which is 2.3 times higher than in roots and flowers (Karawya et al., 1971). The mucilages consist mainly of glucuronic acid, galacturonic acid, galactose, rhamnose, glucose, sucrose, fructose, and trehalose, but uronic acid, fucose, mannose, arabinose, xylose, raffinose, and 2''-O-a-(4-O-methyl-a -d-glucuronosyl)- xylotriose have also been found (Gasparetto et al., 2011). All these sugars are harvested from mucin throughout the gastrointestinal tract by saccharolytic members of the colon microbiota such as bacteroides, *bifidobacteria,* and *lactobacilli* genera (Shen et al., 2011; Bäumler and Sperandio, 2016). These sugars are crucial for the metabolism of colon bacteria as vital carbon sources. Crociani et al. (1994) showed that 96% of *B. bifidum* strains are the only consumers of porcine gastric mucin among 290 bifidobacteria strains tested, and *B. infantis* was the only species that fermented D-glucuronic acid. Consequently, sugars in the mucilage content of the *M. sylvestris* leaf extract could be responsible from the stimulatory effects on the growth of beneficial bacteria except *L. casei* in the present study.

Escherichia coli, *C. perfringens*, and *S. aureus* can often cause foodborne infections and they are associated with gastroenteritis (Ørskov and Ørskov, 1992; Rajkovic, 2014). *Malva sylvestris* leaf extract exhibited a potential inhibitory activity against *E. coli*, *C. perfringens*, and *S. aureus* from 0.25, 2, and 4 mg/ mL concentrations respectively, however it did not inhibit these bacteria completely. Dowek et al. (2020) reported that methanolic extract of *M. sylvestris* leaves showed potential antimicrobial activities against *S. aureus,* a clinical isolate, as that recorded in this study and the zone of inhibition for *S. aureus* was almost 47.2% of the zone for positive control antibiotics. The rate of antibacterial effect was 30.6% relative to negative control for the highest dose in our study. Extract of *M. sylvestris* leaves also did not have an inhibitory activity on *S. aureus* (ATCC 25923) in another study (Azadpour et al., 2016). The extract prepared from the root, leaves, and flowers of *M. sylvestris* at 7.5 mg/mL dose exhibited antibacterial effect of 50% of positive control against a clinical isolate of *S. aureus* while the maximum antibacterial activity was recorded at 15 mg/mL that was almost twice the highest dose (8 mg/mL) used in this study (Walter et al., 2011). The findings about weak antibacterial activity of *M. sylvestris* leaf extract on *E. coli* in the present study are also in accordance with the results of the studies in which *E. coli* strains - without intestinal isolate- was little affected by the methanolic extracts of *M. sylvestris* leaves (Dowek et al., 2020) and resistant to the methanolic extract of *M. sylvestris* aerial parts (Dulger and Gonuz, 2004). On the other hand, there is no report about the effects of *M. sylvestris* extract on *C. perfringens*. However, 1 mg/mL of the extract from the leaves of *M. parviflora*, Egyptian mallow, had a growth inhibition percentage of less than 1% relative to negative control on the same strain of *C. perfringens* used in this study (Omar et al., 2006). The inhibition percentage of the extract on *C. perfringens* was 4.8% relative to negative control in the present study (without statistically significant difference). However, the inhibition percentage of the extract increased to 67.1% at the highest dose of 8 mg/mL.

The other enteropathogen, *F. nucleatum*, is obviously associated with colorectal cancer and promotes the development of colorectal neoplasms (Shang and Liu, 2018). The growth of *F. nucleatum* was inhibited completely by 8 mg/mL of *M. sylvestris* leaf extract in the present study. The ethanolic extract of *M. sylvestris* leaves was also reported to inhibit the same strain of *F. nucleatum* at 1 mg/mL concentration in a previous study (Benso et al., 2015). *Malva sylvestris* leaves were purchased from a local farmer in the northeast Brazil in that study. Phytochemical studies revealed that leaves of *M. sylvestris* are rich in flavonoids and phenolic acids that dominated by luteolin and chlorogenic acid (Terninko et al., 2016) whose antimicrobial effects were reported (Lou et al., 2011; Qian et al., 2020). However, composition of the extracts can vary according to collection location, climatic conditions, soil characteristics, possible differences in the plant genotypes, harvest time, handling types, storage conditions, and extraction method (Fidan et al., 2019). Therefore, the difference between inhibitory concentrations in this and above study (Benso et. al., 2015) might be due to one or more of these factors.

Conclusion

As a conclusion, *M. sylvestris* leaf extract at 0.06-8 mg/mL dose could have favorable effects on colon bacteria since the extract selectively promoted the most of the beneficial species' growth at this dose range while it had a potential inhibitory or inhibitory effect on pathogenic ones. Investigating the effects of *M. sylvestris* leaf extract on other colon bacteria and

testing the *in vivo* effectiveness will contribute to a better understanding of its efficacy on colon microbiota and intestinal health.

Conflict of interest

Author has no conflict of interest to declare.

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