# Antimicrobial efficacy of four different extracts of *Plantago major: An in vitro study*


## ABSTRACT

**Aims:** *Plantago major* is frequently used in traditional treatment for upper respiratory tract diseases such as pneumonia, cough, pharyngitis, and skin, eye, and urinary tract infections. This study aims to evaluate the antimicrobial activity and minimum effective dose of hexane, methanol, ethanol, and water extract of *Plantago major* (PM).
**Study design:** In vitro experimental study.
**Methodology:** *P. major* was crushed into a fine powder and removing dissolved in different solvents (hexane, methanol, ethanol, or water) at the Soxhlet device, then were purified by evaporation of the solvent. All extracts were analyzed for antibacterial and antifungal properties by broth dilution method depending on MIC value determined according to the solvent-microorganism-time trio in DDM.
**Results:** The in vivo test showed that all methods to extract Plantago major have adequate protection against all test microorganisms. Both hexane and water extract showed the same activity on *S. aureus, B. subtilis*, and *P. aeruginosa, C. albicans* and *C. tropicalis* fungi at 4 mg/ml. The lowest activity of PM's hexane and water extract was on *E. coli* and *P. vulgaris* bacteria as 8 mg/ml. The results nominate methanol and ethanol extracts of PM showed higher activity than hexane and water extract. PM ethanol extract showed antimicrobial activity against *Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aerouginosa* at 2ml/uL, and *Bacillus subtilis Escherichia coli* and *Proteus Vulgaris at 4 ml/uL*.
**Conclusion:** The results nominate the Plantago major extract has potential antimicrobials and antifungals. However, the development of antimicrobial agents requires purifying the active bio components by high throughput techniques to achieve effective activity as positive controls.



*Keywords:* *Plantago major, antimicrobial activity, MIC*


## 1. INTRODUCTION

The uses of emerging high throughput technologies allowed us to extract and enrich natural compounds with unique physicochemical properties. Thus, the special attention to active compounds and their derivates of plants increased for natural product-based drug discovery and development.

*Plantago major (P. major)* is a widely used medicinal plant in folk medicine [1]. The plant contains bioactive components such as flavonoids, polysaccharides, terpenoids, lipids, iridoid glycosides, and caffeic acid derivatives [2] [3]. Due to its rich components, the plant was used to treat various medical conditions such as coughs, infection, fever, bleeding, and inflammation [3] [4]. Currently, animal studies focus on using the *Plantago major (PM)* on different medicinal conditions. Parhizgar and colleagues have been demonstrated the protective effect of PM extract in the presence of kidney damage. Parhizgar has shown

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glomerular filtration rate (GFR), urine osmolarity, and urinary excretion rate of potassium were increased via the treatment of PM extract of kidney tissue-damaged rats [5]. In another study, hydroalcoholic extract of *P. major* also had a protective role in doxorubicin-induced nephropathy. These two animal studies have been demonstrated the protective role of PM extract on the renal system. Boskabadi et al. have been shown potent relaxant effects of PM extract on Tracheal Smooth Muscles of rats [6]. The randomized, double-blind placebo-controlled clinical trial done by Jazayeri and colleagues has achieved elevated liver enzymes and a better prognosis for Nonalcoholic Fatty Liver Disease by using 2 gr of PM supplementation twice daily for 12 weeks [7]. Depending on the therapeutic properties shown in both animals and clinical studies, PM extract protects the kidneys and liver against toxicity.

In another way of view, PM extract is not harmful to animals and humans by its protective role, thus potent bioactive compound for drug development. PM extracts were widely used for antibiotics, antioxidants, analgesics, and wound healer purposes from ancient times. The antibacterial activity of PM extract was evaluated by Sharma et al. They found no activity for periodontal pathogens by the Kirby-Bauer disc diffusion technique [8]. However, none of the studies were performed to evaluate PM extract's antimicrobial activity by MIC method against standard test microorganisms. Thus, this study investigated the bioactivity of a Plantago major against pathogens: *Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus subtilis, Pseudomonas aeruginosa*, *Proteus Vulgaris* as antimicrobial, and *Candida albicans* and *Candida tropicalis* as antifungal.

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**Material and Method**

## 2.1 Chemicals

The medium used for this study was Nutrient Broth for bacterial, and RPMI-1640 medium with L-glutamine for fungi were purchased from Sigma-Aldrich (Hamburg, Germany). The microbial lines were purchased from American Type Culture Collection (ATCC®, Manassas, VA, USA). The antibiotics and antifungal pharmaceuticals were used as a positive control as follows: Ampicillin (Mustafa Nevzat Pharmaceuticals, Turkey); Gentamicin (Bilim Pharmaceuticals, Turkey); Fluconazole (Pfizer Pharmaceuticals, Roerig Division (New York, N.Y.). All other analytical grade chemicals used without further purification were purchased from Sigma-Aldrich (Hamburg, Germany).

## 2.2 Preparation of Plant Extract

The plants were washed with distilled water, dried in the shade with continuous airflow, and then grounded into 1-3 mm pieces. The powder (20 mg) was then macerated with 200 ml solution for eight hours in Soxhlet (Wisd, Wise Therm). After this process, the plant was dried in an oven to remove its solvent, and then the plant sample was treated with a second solvent with higher polarity. The extraction step was done with four different solvents as follows: hexane ($C_6H_{14}$, $T_B$:69ºC), methanol ($CH_3OH$, $T_B$:64,7 ºC), ethanol ($C_2H_5OH$, $T_B$: 78.4ºC and water ($H_2O$, $T_B$:100ºC). After this step, all extracts were evaporated at 50 ºC to obtain a solvent-free extract, which will be stored in the refrigerator (0-4 ºC) until experiments. All of them were sterilized by a membrane filter (0.2 µm) before use [9].

## 2.3 Antimicrobial and Antifungal Activity Assay

Antimicrobial activity of extracts was applied to Gram-positive bacteria cultures: *Staphylococcus aureus* (ATCC 6538), *Enterococcus faecalis* (ATCC 292112), *Bacillus*

*subtilis* (ATCC 6633), whereas gram-negative bacterial cultures *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (27853), *Proteus Vulgaris* (ATCC 13315). Antifungal activity has been studied against *Candida albicans* (ATCC 60193) and *Candida tropicalis* (ATCC 13803).

Antimicrobial activity analyses of four different extracts of *Plantago major* were performed using Broth microdilution methods as recommended by Clinical & Laboratory Standards Institute [10-11]. In brief, the microorganism inoculum was prepared by 18 hours of fresh incubated microbial cultures adjusted with turbidity, which means a final concentration 1.5 × 108 CFU/mL. These microorganisms were pipetted in each well. The plant extracts were dissolved in dimethyl sulfoxide (DMSO), and after two times concentrated extracts at 32-0,156 mg/ml concentration were pipetted in each well of microtiter plates. The wells were filled with 100 µL culture suspension as Nutrient broth (Sigma-Aldrich, Germany). for antimicrobial activity and RPMI-1640 medium with L-Glutamin (Sigma-Aldrich, Germany) for antifungal activity as a growth medium. The standard antibiotics and fungicide were used as Ampicillin, Gentamicin, and Fluconazole 2 µg/ml, respectively. The aerobic incubation condition was applied at 37 °C 18-24 h for bacteria and 48 h for fungi. The minimum inhibitory concentration (MIC) was calculated by visible inhibition of the microbial growth at the lowest concentration.

## 3. RESULTS AND DISCUSSION

Table 1 is summarized the antibacterial and antifungal effects of different extracts of *Plantago Major.* The antimicrobial activity of hexane and aqueous extracts of PM against all test microorganisms except *Enterococcus faecalis* was the same.

**Tablo 1 Antimicrobial activity of different extracts of *Plantago Major***

| MIC (mg/ml) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Test Materials** | **Bakteriler** | | | | | | **Fungi** | |
| | **Gram (+) Bacteria** | | | **Gram (-)Bacteria** | | | | |
| | ***Staphylococcus aureus* ATCC 6538** | ***Enterococcus faecalis* ATCC 292112** | ***Bacillus subtilis* ATCC 6633** | ***Escherichia coli* ATCC 25922** | ***Pseudomonas aerouginosa* ATCC 27853** | ***Proteus vulgaris* ATCC 13315** | ***Candida albicans* ATCC 60193** | ***Candida tropicalis* ATCC 13803** |
| PM-nHexOH | 4 | 2 | 4 | 8 | 4 | 8 | 4 | 4 |
| PM-MeOH | 2 | 2 | 4 | 4 | 2 | 4 | 2 | 4 |
| PM-EtOH | 2 | 2 | 4 | 4 | 2 | 4 | 2 | 4 |
| PM-H2O | 4 | 4 | 4 | 8 | 4 | 8 | 4 | 4 |
| **Gentamicin** | $10^{-3}$ | $10^{-3}$ | $8.10^{-3}$ | $16.10^{-3}$ | $8.10^{-3}$ | $16.10^{-3}$ | - | - |
| **Ampicillin** | $16.10^{-3}$ | $16.10^{-3}$ | $16.10^{-3}$ | $32.10^{-3}$ | $32.10^{-3}$ | $16.10^{-3}$ | - | - |
| **Fluconazole** | - | - | - | - | - | - | $625.10^{-3}$ | $25.10^{-1}$ |
| **DMSO** | - | - | - | - | - | - | - | - |

The hexane extract showed the highest activity with a 2 mg/mL dose against the bacteria *E. faecalis*, and it was found 4 mg/ml in water extract. Both hexane and water extract showed the same activity on *S. aureus, B. subtilis, and P. aeruginosa* bacteria and *C. albicans* and

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*C. tropicalis* fungi as 4 mg/ml. The lowest activity of PM's hexane and water extract was on *E. coli* and *P. vulgaris* bacteria as 8 mg/ml (Table 1).

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The results revealed that the efficiency of both ethanol and methanol extracts of PM was similar to all test microorganisms. Methanol and ethanol showed the highest activity (2mg/mL) in *S. aureus, E.faecialis and P.aeruginosa* bacteria and *C.albicans* yeast however had low activity on *B.subtilis, E.coli P.vulgaris bacteria,* and *C.tropicalis* yeast.

In addition, the results (table1) show that methanol and ethanol extracts showed higher activity than hexane and water extract. It is known that hexane has polar solubility, methanol and ethanol have semi-polar, and water has apolar solubility. These differences explain that ethanol and methanol would be better solvents for PM, and bioactive compounds were more easily resolved in ethanol and methanol. The four types of PM extracts exhibited inhibitory effects against test microorganisms; however, these antimicrobial activities were below the MIC value of positive controls (ampicillin gentamicin and fluconazole). thus, we can conclude that PM extracts show antimicrobial activity but are not as effective as standard therapeutics.

This study provides further documentation of the applicability of the *Plantago major* extract against microorganisms. Previous studies have shown that PM has no antimicrobial activity against primary plaque colonizers or periodontal pathogens [8]. However, we clearly showed that PM ethanol extract showed antimicrobial activity against *Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aerouginosa* at 2ml/uL, and *Bacillus subtilis Escherichia coli* and *Proteus Vulgaris* at 4 ml/uL. In addition to ethanol extract, hexane, methanol, and water extract showed antimicrobial activity. Together with this, all extracts showed antifungal activity against *Candida albicans* and *Candida tropicalis.* In a previous study, the Kirby-Bauer disc diffusion technique was used to investigate the antimicrobial activity, which is not recommended as a reference method.

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In contrast, we clearly showed antimicrobial activity by the MIC method, which is a gold standard used in clinics to calculate the antimicrobial activity at routine practice. In addition, Sharma and colleagues prepared their text extract with maceration; thus, the preparation procedure should affect the concentration of bioactive compounds in total. Similar to our study, Ferrazzano and colleagues demonstrated a significant antimicrobial effect of *Plantago lanceolata* and evaluated the plant extract as a natural anti-cariogenic agent [12]. Although *Ferrazzano* et al. studied different species, both plants have the same genus and have similar phytochemicals. The broth dilution MICs for Planto major extracts tested against *C. Albicans* and C. *tropicalis* showed significant inhibition, which means PM extracted by different solvents might be used with its antifungal activity. In summary, we have provided documentation of PM extracts with antimicrobial and antifungal activity; therefore, PM extracts should be a good candidate for drug development.

## 4. CONCLUSION

This study demonstrated the antimicrobial activity of Plantago major against all test microorganisms. In addition, n-EtOH and n-MeOH extract of PM showed better antimicrobial and antifungal activity when compared to hexane and aqueous extracts. The difference in antimicrobial efficiency at different extracts clearly showed that the extraction method is important to obtain bioactive molecules. Despite significant improvements in synthetic molecules in the pharmaceutical industry, the growing antibiotic resistance problem still requires finding alternative antimicrobial agents. In this frame, the discovery of plant-based antibiotics is also essential to reduce the side effects of synthetic antimicrobials. Thus, we recommended testing different extract types and using gold standards as antimicrobial

activity determination. The results of our study show that PM extracts are effective as an antimicrobial agent. However, the active component of the plant extracts should be enhanced, or active components should be purified by high-throughput techniques to develop bio-based pharmaceuticals.

**COMPETING INTERESTS DISCLAIMER:**

**AUTHORS HAVE DECLARED THAT NO COMPETING INTERESTS EXIST. THE PRODUCTS USED FOR THIS RESEARCH ARE COMMONLY AND PREDOMINANTLY USE PRODUCTS IN OUR AREA OF RESEARCH AND COUNTRY. THERE IS ABSOLUTELY NO CONFLICT OF INTEREST BETWEEN THE AUTHORS AND PRODUCERS OF THE PRODUCTS BECAUSE WE DO NOT INTEND TO USE THESE PRODUCTS AS AN AVENUE FOR ANY LITIGATION BUT FOR THE ADVANCEMENT OF KNOWLEDGE. ALSO, THE RESEARCH WAS NOT FUNDED BY THE PRODUCING COMPANY RATHER IT WAS FUNDED BY PERSONAL EFFORTS OF THE AUTHORS.**

**REFERENCES**


1. Wang H, Zhao C, Huang Y, Wang F, Li Y, Chung HY. Chemical Constituents and Bioactivities of Plantaginis Herba. Hong Kong Med J. 2015;22:29–35.

2. Samuelsen AB. The traditional uses, chemical constituents and biological activities of Plantago major L. A review. J Ethnopharmacol. 2000;71:1–21.

3. Najafian Y, Hamedi SS, Farshchi MK, Feyzabadi Z. Plantago major in Traditional Persian Medicine and modern phytotherapy: a narrative review. Electron Physician. 2018;10(2):6390-9.

4. Ghanadian M, Soltani R, Homayouni A, Khorvash F, Jouabadi SM, Abdollahzadeh M. The Effect of Plantago major Hydroalcoholic Extract on the Healing of Diabetic Foot and Pressure Ulcers. A Randomized Open-Label Controlled Clinical Trial. Int J Low Extrem Wounds 2022; (In press).

5. Parhizgar S, Hosseinian S, Soukhtanloo M, Bideskan AE, Hadjzadeh MA, Shahraki S et al. Plantago major protects against cisplatin-induced renal dysfunction and tissue damage in rats. Saudi J Kidney Dis Transpl. 2018;29(5):1057-64.

6. Boskabadi J, Saadat S, Boskabady MH. The Relaxant Effect of Plantago Major on Rat Tracheal Smooth Muscles and Its Possible Mechanisms. Iran J Allergy Asthma Immunol. 2020;19(4):386-96.

7. Jazayeri SF, Ghods R, Hashem Dabaghian F, Shojaii A, Moravej SAA, Khadem E et al. A Randomized Double-Blind Clinical Trial. Evid Based Complement. Alternat Med 2021;6693887.

8. Sharma H, Yunus GY, Mohapatra AK, Kulshrestha R, Agrawal R, Kalra M. Antimicrobial efficacy of three medicinal plants Glycyrrhiza glabra, Ficus religiosa, and Plantago major on inhibiting primary plaque colonizers and periodontal pathogens: An in vitro study. Indian J Dent Res. 2016;27(2):200-4.

9. Kaur K, Arora S, Kumar S, Nagpal A.. Antimutagenic Activities of Acetone and Methanol Fractions of Terminalia arjuna. Food and Chemical Toxicology. 2002;40:1475-82.

10. CLSI, Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts, Approved Guideline. CLSI document M44-A. CLSI, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA; 2004..

11. CLSI, Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard-Ninth Edition. CLSI Document M07-A9. Wayne, Pennsylvania. 2012b.

12. Ferrazzano GF, Cantile T, Roberto L, Ingenito A, Catania MR, Roscetto E et al. Determination of the in vitro and in vivo antimicrobial activity on salivary Streptococci and Lactobacilli and chemical characterisation of the phenolic content of a Plantago lanceolata infusion. Biomed Res Int. 2015;286817.

## DEFINITIONS, ACRONYMS, ABBREVIATIONS

Here is the Definitions section. This is an optional section.
$\mathbf{T_B}$: Boiling point
**PM:** *Plantago Major*