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Evaluation of antimicrobial and antibiofilm activities of some *Phytolacca americana* L. extracts

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ABSTRACT: Antimicrobial resistance remains a significant global challenge across various fields. To solve this problem, researchers are developing antimicrobial agents, antiseptics, and disinfectants with different formulations. In this study, methanol (MeOH) extracts of *Phytolacca americana* L. fruits and leaves, were evaluated for their both antimicrobial and antibiofilm activities using the microdilution method and antibiofilm assay against bacteria and fungi including *Staphylococcus aureus*, *Streptococcus mutans*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Candida albicans*, *C. parapsilosis*, *C. krusei*, *Trichophyton rubrum*, *Epidermophyton floccosum*, and *Microsporum gypseum*. According to the activity studies, extracts displayed 32-128 µg/mL MICs against tested bacteria and fungi. In the microdilution method, inhibition curves of IC_{50} obtained by the resazurin microplate assay for the extracts showed results between 12-41 µg/mL.

KEYWORDS: Antimicrobial; antibiofilm; broth microdilution; colorimetric microdilution; Phytolacca; disinfectant; pharmaceutical agents.

1. INTRODUCTION

Biofilm is a type of extracellular polymeric matrix that allows bacteria, archaea, yeasts, molds, algae and protozoa to adhere to each other or surfaces [3]. This extracellular polymeric substance matrix, which we call EPS (extracellular polymeric substance), consists of extracellular DNA (eDNA), polysaccharides, proteins and glycolipids. Biofilms can develop on almost any surface, posing a major threat to human health. It has been reported that the majority of hospital infections are related to the colonization of pathogens on the surfaces of implanted medical devices such as respiratory devices, catheters (central venous, urinary), prosthetic heart valves, and orthopedic devices [4]. Biofilm-induced antibiotic resistance has become an important problem in the treatment of chronic infections [5-7]. It has been reported that different strategies should be developed in the treatment of chronic and acute infections that develop as a result of biofilm formation [8-11]. Necessary measures should be taken to prevent biofilm formation. Due to the increasing incidence of biofilm-induced infections, researchers are putting significant efforts into controlling and preventing biofilm formation [12-14].

P. americana, commonly known as pokeweed, is a perennial plant native to North America. It is a plant that blooms between June and September and lives in fields, hillsides and shrub habitats. It is a perennial herb belonging to the Phytolaccaceae family and is native to North America. It has a strong and deep root system that prevents it from being affected by fires, so it can continue to live by regrowing from its roots after a fire. The root is used as a traditional diuretic, in the treatment of rheumatoid arthritis and flatulence, despite its strong toxicity. At the same time, although it is stated that *P. americana* taxon is poisonous and should be used with caution, it has also been reported that it is used as treatment, food and animal feed, especially in China [15]. It has been reported that *P. americana* is widely used in traditional medicine in South and Central America for the treatment of many diseases due to its antimicrobial, anti-inflammatory, anticancer and stimulant effects [16].

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Recent studies have mainly focused on types on methanol extracts of *P. americana* (fruit, leaves) antimicrobial and antibiofilm activities against bacteria (*S. aureus*, *S. mutans*, *B. subtilis*, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *A. baumannii*), yeast like fungi (*C. albicans*, *C. parapsilosis*, *C. krusei)* and dermatophytes (*T. rubrum*, *E. floccosum, M. gypseum*).

2. RESULTS

Antimicrobial resistance is one of the biggest problems worldwide. It is clear there is still a need for antibiofilm active ingredients. Infections caused by biofilm-producing strains require more comprehensive treatments and this issue still maintains its importance. At this point, alternative strategies and new antibiofilm agents are needed against antibiotic-resistant biofilm communities. Various compounds obtained from natural sources have been proven to be beneficial as antimicrobial agents, antiseptics and disinfectants. In the present study, methanol extracts of *P. americana* (fruits, leaves) were evaluated for their *in vitro* antimicrobial and antibiofilm activities nearby MBS/MBC, MFS/MFC effects against standard strains of *S. aureus*, *S. mutans*, *B. subtilis*, *E. faecalis, E. coli, P. aeruginosa, K. pneumoniae*, *A. baumannii*, C. *albicans*, *C. parapsilosis*, *C. krusei*, *T. rubrum*, *E. floccosum*, and *M. gypseum*. According to [17]. *P. americana* has anti-inflammatory, anti-mutagenic, antioxidant, antibacterial, and anticancer properties. In a study conducted with crude extract, a strong activity (100% growth inhibition) was observed against *Porphyromonas gingivalis* and a moderate effect (44% growth inhibition) was determined against *S. mutans* at 1.8 µg/mL [2]. In another study, the antimicrobial activity of *P. americana* was reported against *Malassezia furfur* and *Vibrio parahaemolyticus*; it has been reported to be higher than *E. coli* and *Staphylococcus epidermidis*. However, antimicrobial activity against *V. parahaemolyticus* was not observed in all parts of *P. americana*. The antimicrobial activity was the highest in the leaf compared to the different plant parts. The antimicrobial activity of *P. americana* was found to vary depending on the bacterial strains and the different plant parts used. *P. americana* extracts showed higher antimicrobial activity on fungi and Gram-positive bacteria than Gram-negative bacteria [18]. A recent work assessed the phenolic contents, antibacterial, antibiofilm, and anticancer properties of methanol extracts of *P. americana* fruit (exocarp, pulp, and berry juice) and seed. The seed extract was discovered to possess stronger antibacterial activity than other parts [19]. In the present study, higher antimicrobial activity was seen for *P. americana* (leaves, fruits-MeOH) at MIC values of 32 against *B. subtilis* as well as better antimicrobial activity was detected for *P. americana* (leaves- MeOH) at MIC values of 32 µg/mL against *K. pneumoniae* and *A. baumannii*. The antifungal activity of the extracts was better against *C. albicans* at MIC values of 64 µg/mL, and the antidermatophytic activity was better against *T. rubrum* at MIC values of 32 µg/mL (**Table 1**). Currently, there are many extracts known to have antimicrobial activity, but there are few literature records on the antimicrobial activity of *P. americana* according to the literature searches. It is not surprising that the antimicrobial activities of plant species differ due to phytochemical profile. The antimicrobial activity of *P. americana*, therefore, can be attributed to the presence of bioactive compounds such as saponins, alkaloids, and flavonoids [20, 21].

In the present study, the antimicrobial activity of methanol extracts from the leaves and fruits of *P. americana* was evaluated within the concentration ranges of MIC (32-256 µg/mL) and IC_{50} (12-41 µg/mL). The results of antibiofilm activity determined for *P. americana* MeOH extracts are important because they include the first *in vitro* research results obtained for their use as both antiseptic and antimicrobial agent and direct further studies as new active ingredients (**Figure 1,2,3,4**).

P. americana can be beneficial in developing alternative antimicrobial therapies. However, it is important to keep in mind that while *P. americana* shows bioactivity, its use must be approached with caution due to the toxic nature of some compounds, including the lectins and saponins, which can be harmful if not properly prepared or dosed. Despite the fact that there are several studies on *P. americana*, little is known about how it works against infectious pathogens. More excellent research is needed to prove the investigated plants' clinical effectiveness. Research examining the mode of action may also support the antimicrobial activity of *P. americana*.

Table 1. Antibacterial activity of the extracts as MICs; in µg/mL as well as MBS/MBC; µg/mL, and MFS/-MFC effect nearby IC_{50} values of extracts against biofilm formation.

	S. aureus ATCC 25923				*S. mutans* ATCC 21752			
Extracs	**MIC_a**	**MIC_b**	**MBS/MBC**	**IC_{50}**	**MIC_a**	**MIC_b**	**MBS/MBC**	**IC_{50}**
P. americana (fruits- MeOH)	64	128	≥512/-	22±0.64	64	64	≥512/-	24±1.85
P. americana (leaves- MeOH)	64	64	≥512/-	20±0.45	128	128	≥512/-	38±2.35
AMP	0.5				<0.12			
LFX	0.25				0. 5			
	***B. subtilis* ATCC 6633**				***E. faecalis* ATCC 29212**			
Extracs	**MIC_a**	**MIC_b**	**MBS/MBC**	**IC_{50}**	**MIC_a**	**MIC_b**	**MBS/MBC**	**IC_{50}**
P. americana (fruits- MeOH)	32	32	≥512/-	21±0.23	64	64	≥512/-	38±2.40
P. americana (leaves- MeOH)	32	32	≥512/-	18±0.26	64	64	≥512/-	41±2.24
AMP	0.12				0.23			
LFX	-				0.5			
	***E. coli* ATCC 25922**				***P. aeruginosa* ATCC 10145**			
Extracs	**MIC_a**	**MIC_b**	**MBS/MBC**	**IC_{50}**	**MIC_a**	**MIC_b**	**MBS/MBC**	**IC_{50}**
P. americana (fruits- MeOH)	64	64	≥512/-	25±3.46	64	64	≥512/-	34±1.95
P. americana (leaves- MeOH)	64	64	≥512/-	28±2.24	64	64	≥512/-	34±2.55
AMP	0.5				<0.12			
LFX	0.25				0. 5			
	***K. pneumoniae* RSKK 574**				***A. baumannii* RSKK 02026**			
Extracs	**MIC_a**	**MIC_b**	**MBS/MBC**	**IC_{50}**	**MIC_a**	**MIC_b**	**MBS/MBC**	**IC_{50}**
P. americana (fruits- MeOH)	64	64	≥512/-	34±3.48	64	64	-/512	40±0.37
P. americana (leaves- MeOH)	32	32	≥512/-	20±0.38	32	32	-/512	24±0.62
AMP	0.12				0.23			
LFX	-				0.5			
	***C. albicans* ATCC 10231**				***C. parapsilosis* ATCC 90028**			
Extracs	**MIC_a**	**MIC_b**	**MBS/MBC**	**IC_{50}**	**MIC_a**	**MIC_b**	**MBS/MBC**	**IC_{50}**
P. americana (fruits- MeOH)	64	64	≥512/-	12±7.80	256	256	≥512/-	15±4.25
P. americana (leaves- MeOH)	64	64	≥512/-	13±4.60	256	256	≥512/-	15±4.66
KET	2				2			
FLU	4				4			
	***C. krusei* ATCC 6258**				***T. rubrum* RSKK 486**			
Extracs	**MIC_a**	**MIC_b**	**MBS/MBC**	**IC_{50}**	**MIC_a**	**MIC_b**	**MBS/MBC**	**IC_{50}**
P. americana (fruits- MeOH)	256	256	≥512/-	19±10.99	32	32	≥512/-	12±1.74
P. americana (leaves- MeOH)	256	256	≥512/-	18 ±5.02	32	32	≥512/-	13±1.90
ITRA	-				0.25			
GRI	-				0.5			
TER	-				0.125			
KET	4				2			
FLU	64				-			

Extracs	*E. floccosum* RSKK 3027				*M. gypseum* NCPF 580			
	MIC_a	MIC_b	MBS/MBC	IC_{50}	MIC_a	MIC_b	MBS/MBC	IC_{50}
P. americana (fruits- MeOH)	128	128	≥512/-	15±3.09	128	128	≥512/-	19±2.29
P. americana (leaves- MeOH)	128	128	≥512/-	15±1.90	128	128	≥512/-	18±3.89
ITRA	0.25				0.25			
GRI	0.5				0.5			
TER	0.125				0.125			

Extracts: *Phytolacca americana* fruits-MeOH, *Phytolacca americana* leaves-MeOH. **AMP**: Ampicillin, **LFX**: Levofloxacin. **GEN**: Gentamicin, **ITRA**: Itraconazole, **GRI**: Griseofulvin, **TER**: Terbinafine, **FLU**: Fluconazole, **KET**: Ketoconazole, **ATCC**; American Type Culture Collection, ***S. aureus***: *Staphylococcus aureus*, ***S. mutans***: *Streptococcus mutans*, ***E. faecalis***: *Enterococcus faecalis*, ***C. albicans***: *Candida albicans*, ***C. parapsilosis***: *Candida parapsilosis*, ***C. krusei***: *Candida krusei*, ***T. rubrum***: *Trycophyton rubrum*, ***E. floccosum***: *Epidermophyton floccosum*, ***M. gypseum***: *Microsporum gypseum*, **MFS/-MFC**: Minimum fungistatic/-cidal, IC_{50}, values of inhibition concentration against biofilm formation. **RSKK;** culture collection of Turkish Public Health Institution formerly; Refik Saydam Central Hygiene Institute, **MICa:** Minimum Inhibition Concentration **MICb:** MICb MICs of each sample material that prevented color change (Resazurin).

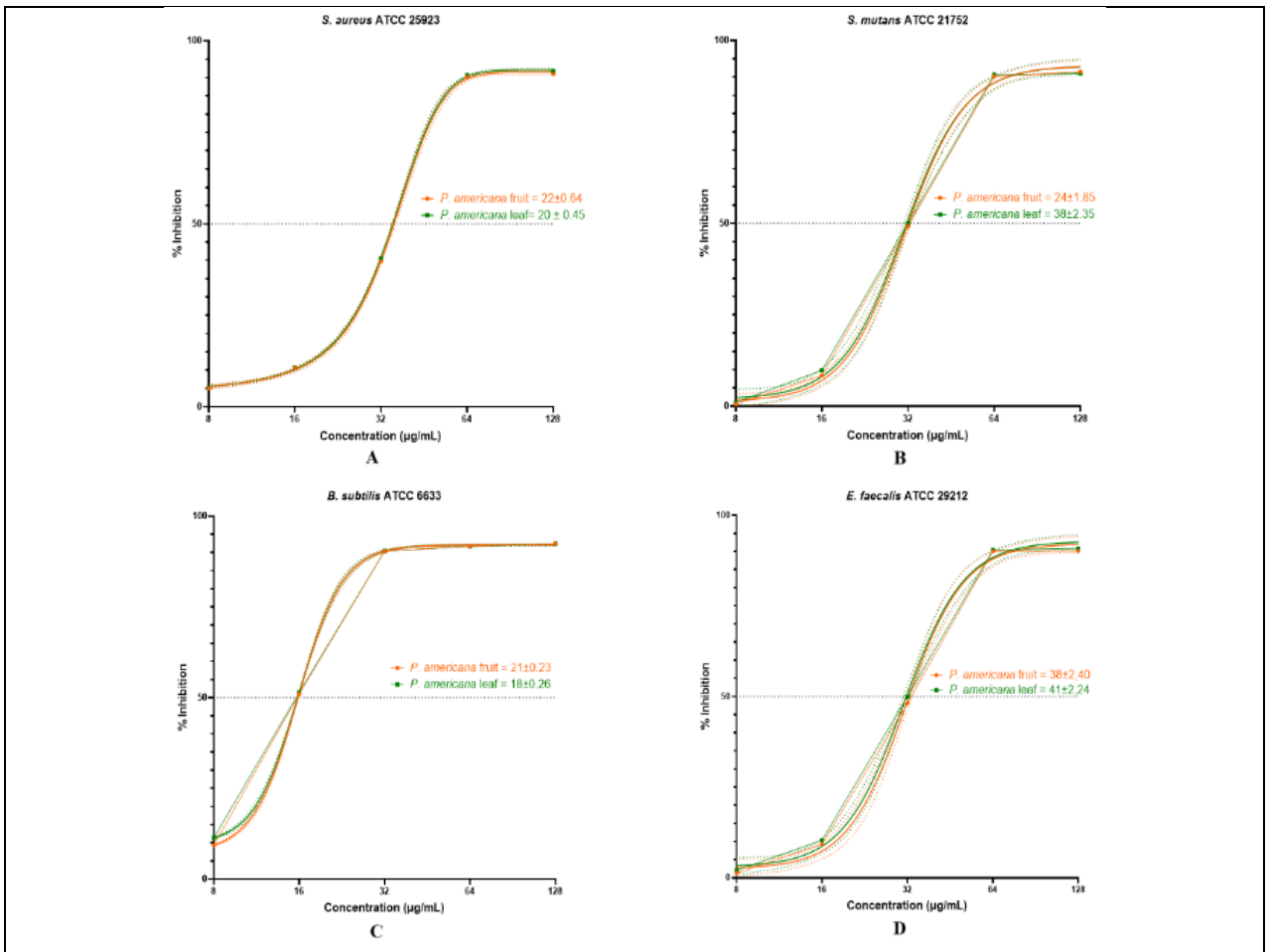


Fig.1 Concentration-related curves for % inhibitions of tested bacteria by extracts A: *S. aureus*; B: *S. mutans*; C: *B. subtilis*; D: *E. faecalis*.

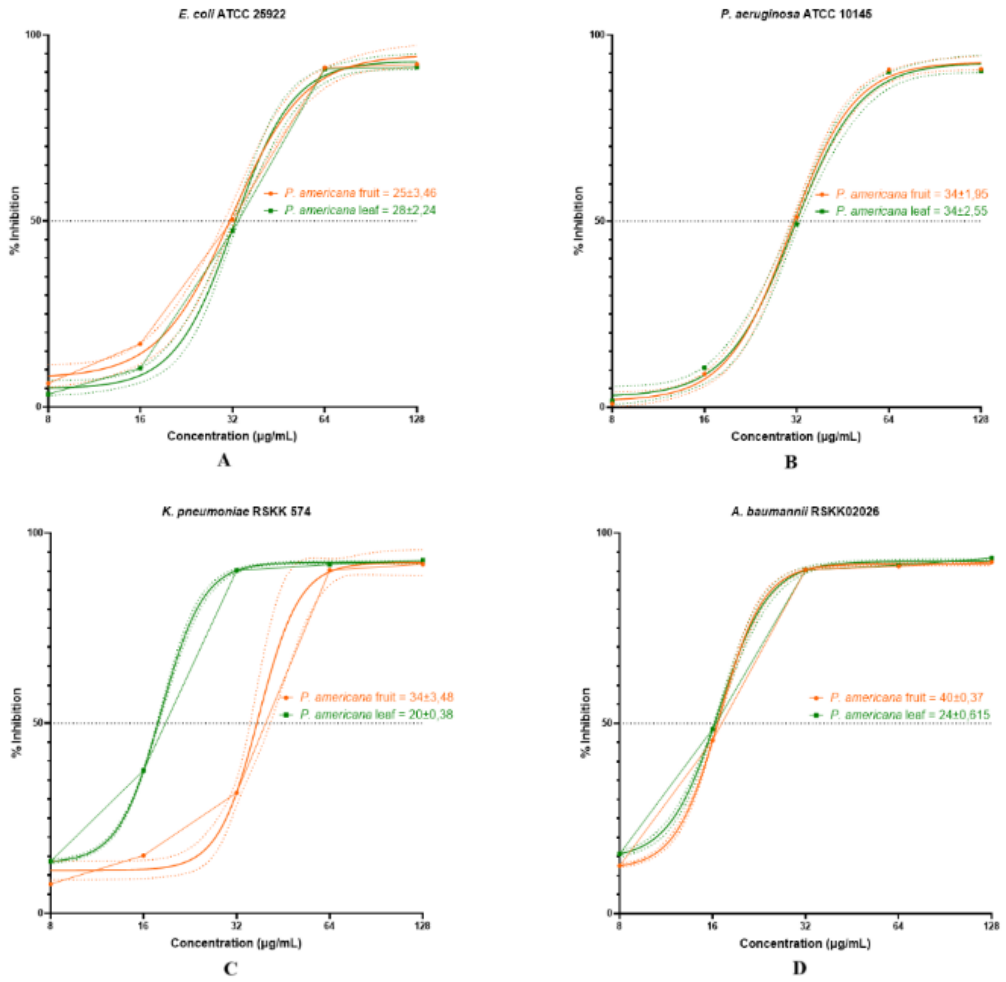
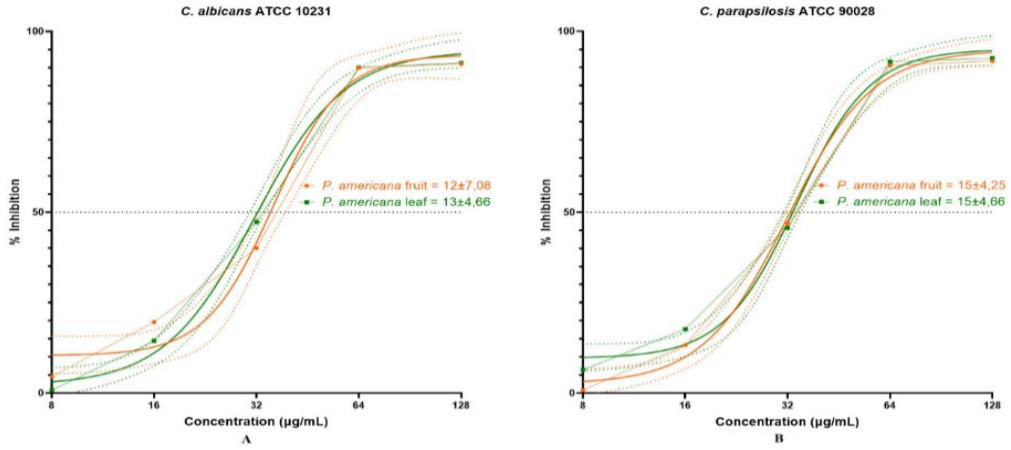


Fig. 2 Concentration-related curves for % inhibitions of tested bacteria by extracts A: *E. coli*; B: *P. aeruginosa*; C: *K. pneumonia*; D: *A. baumannii*.



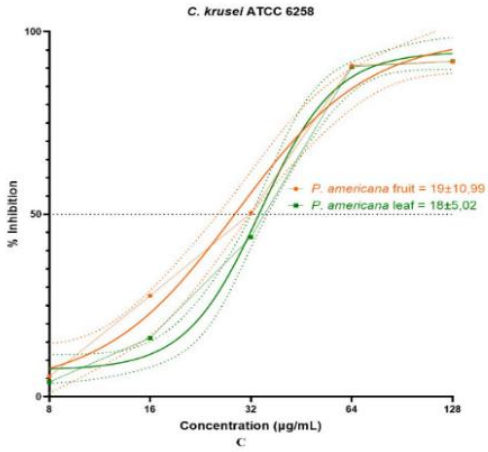


Fig. 3. Concentration-related curves for % inhibition of tested yeast like fungi by extracts A: *C. albicans*; B: *C. parapsilosis*; C: *C. krusei.*

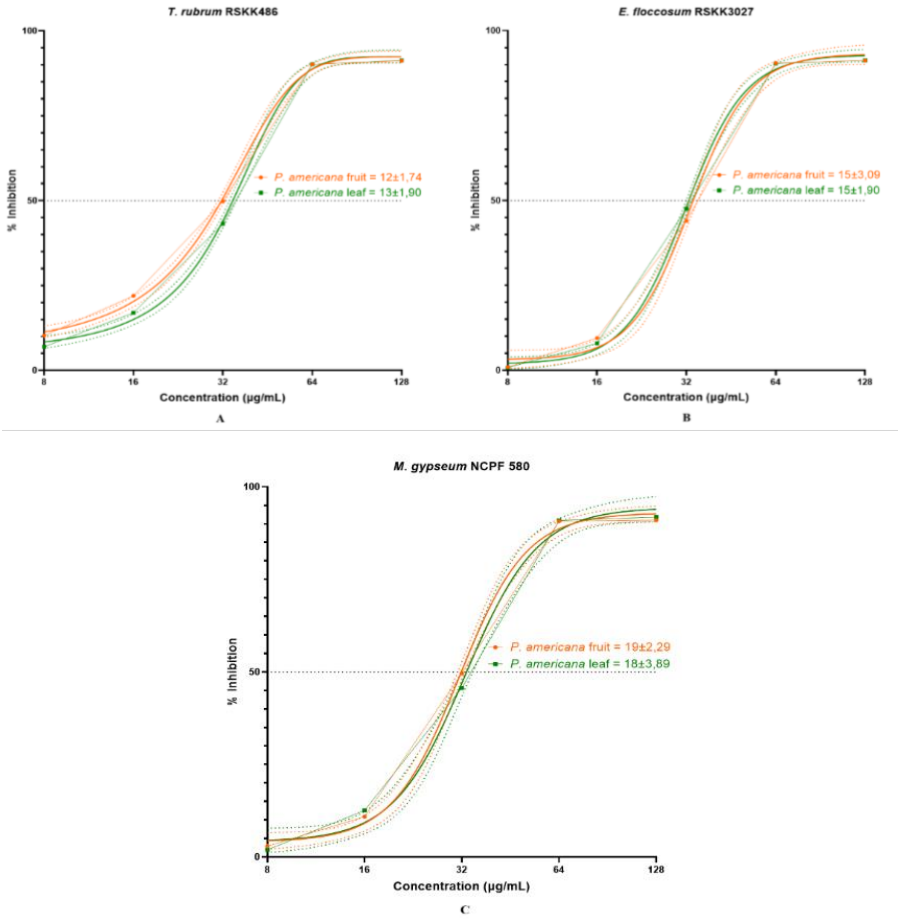


Fig. 4 Concentration-related curves for % inhibitions of tested dermatophytes by extracts A: *T. rubrum*; B: *E. floccosum*; C: *M. gypseum.*

3. DISCUSSION

Antimicrobial resistance is one of the biggest problems worldwide. It is clear there is still a need for antibiofilm active ingredients. Infections caused by biofilm-producing strains require more comprehensive treatments and this issue still maintains its importance. At this point, alternative strategies and new antibiofilm agents are needed against antibiotic-resistant biofilm communities. Various compounds obtained

from natural sources have been proven to be beneficial as antimicrobial agents, antiseptics and disinfectants. In the present study, methanol extracts of *P. americana* (fruits, leaves) were evaluated for their *in vitro* antimicrobial and antibiofilm activities nearby MBS/MBC, MFS/MFC effects against standard strains of *S. aureus*, *S. mutans*, *B. subtilis*, *E. faecalis, E. coli, P. aeruginosa, K. pneumoniae*, *A.* baumannii, C. *albicans*, *C. parapsilosis*, *C. krusei*, *T. rubrum*, *E. floccosum*, and *M. gypseum*. According to [17]. *P. americana* has anti-inflammatory, anti-mutagenic, antioxidant, antibacterial, and anticancer properties. In a study conducted with crude extract, a strong activity (100% growth inhibition) was observed against *Porphyromonas gingivalis* and a moderate effect (44% growth inhibition) was determined against *S. mutans* at 1.8 µg/mL [2]. In another study, the antimicrobial activity of *P. americana* was reported against *Malassezia furfur* and *Vibrio parahaemolyticus*; it has been reported to be higher than *E. coli* and *Staphylococcus epidermidis*. However, antimicrobial activity against *V. parahaemolyticus* was not observed in all parts of *P. americana*. The antimicrobial activity was the highest in the leaf compared to the different plant parts. The antimicrobial activity of *P. americana* was found to vary depending on the bacterial strains and the different plant parts used. *P. americana* extracts showed higher antimicrobial activity on fungi and Gram-positive bacteria than Gram-negative bacteria [18]. A recent work assessed the phenolic contents, antibacterial, antibiofilm, and anticancer properties of methanol extracts of *P. americana* fruit (exocarp, pulp, and berry juice) and seed. The seed extract was discovered to possess stronger antibacterial activity than other parts [19]. In the present study, higher antimicrobial activity was seen for *P. americana* (leaves, fruits-MeOH) at MIC values of 32 against *B. subtilis* as well as better antimicrobial activity was detected for *P. americana* (leaves- MeOH) at MIC values of 32 µg/mL against *K. pneumoniae* and *A. baumannii*. The antifungal activity of the extracts was better against *C. albicans* at MIC values of 64 µg/mL, and the antidermatophytic activity was better against *T. rubrum* at MIC values of 32 µg/mL. Currently, there are many extracts known to have antimicrobial activity, but there are few literature records on the antimicrobial activity of *P. americana* according to the literature searches. It is not surprising that the antimicrobial activities of plant species differ due to phytochemical profile. The antimicrobial activity of *P. americana*, therefore, can be attributed to the presence of bioactive compounds such as saponins, alkaloids, and flavonoids [20, 21].

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4. CONCLUSION

In our research, the antibiofilm active concentrations (IC_{50}: 12-41 µg/mL) determined in methanol extracts of fruits and leaves of Phytolacca species, *Phytolacca americana* L., have provided a strong source of evidence that they can be used as antiseptics in this dose range if supported by *ex vivo*/*in vivo* studies on tissue.

5. MATERIALS AND METHODS

5.1. Medicinal Plant Extracts

The fruits and leaves of *P. americana* used in the study were collected from Ermenek, Karaman (1350 m) in August 2020. 2021. The identification of the plants carried out by Prof. Dr. Osman TUGAY from the Department of Pharmaceutical Botany, Faculty of Pharmacy, Selçuk University. A voucher specimen (KNYA-30.213) was deposited at the Herbarium of the Faculty of Science, Selcuk University, Konya, Türkiye. Ten grams of air-dried and grounded fruits and leaves of the plant were macerated with 200 mL of methanol. Then, the extracts were filtered, and fresh methanol was added. These solvent extraction processes were repeated three times (24 h x 3). The combined extracts were evaporated to dryness using a

rotary evaporator at 40°C to give 2.6 g of *P. americana* fruits-MeOH and 2.3 g of *P. americana* leaves-MeOH. The extracts were kept at 4°C until required.

5.2. Antimicrobial activity

P. americana fruits-MeOH, and *P. americana* leaves-MeOH were dissolved in dimethylsulphoxide (30%) and H_2O (70%) at a final concentration of 1024 µg/mL and the solutions were sterilized by filtration using 0.22 mm Millipore (MA 01730, USA) and used as the stock solutions. Ampicillin (AMP), gentamicin (GN), levofloxacin (LFX), fluconazole (FLU), ketoconazole (KET), terbinafine (TER), griseofulvin (GRI), itraconazole (ITRA) were used as the standard antibacterial and antifungal drugs. Reference antibacterial agents were purchased from Sigma Chemical Co. and dissolved in phosphate buffer solution (ampicillin, pH 8.0; 0.1 mol/mL), dimethylsulphoxide (ketoconazole, terbinafine, griseofulvin, itraconazole) or in water (gentamicin, levofloxacin, fluconazole). The stock solutions of the agents were prepared in the medium as described previously. Antimicrobial activity tests were carried out against standards (ATCC; American type culture collection, RSKK; culture collection of Turkish Public Health Institution formerly; Refik Saydam Central Hygiene Institute, NCPF; National Collection of Pathogenic Fungi), Gram-positive standard strains of *S. aureus* ATCC 292013, *S. mutans* ATCC 21752, *B. subtilis* ATCC 10231, Gram-negative standard strains of *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *K. pneumoniae* RSKK 574, *A. baumannii* RSKK 02026, yeast like fungi of *C. albicans* ATCC 10231, *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258, dermatophytes fungi; *T. rubrum* (RSKK 486), *E. floccosum* (RSKK 3027) and *M. gypseum* (NCPF 580) were used for the determination of anti-biofilm activity using broth microdilution assay. Mueller Hinton Broth (MHB; Difco), Mueller Hinton Agar (MHA; Oxoid) were used for growing and diluting bacterial suspensions, nearby Mitis Salivarius agar/-broth (Difco Laboratories, Detroit, MI) supplemented with sucrose and bacitracin for *S. mutans* were used. The synthetic medium RPMI-1640 with L-glutamine was buffered to pH: 7 with 3-[N-morpholino]-propansulfonic acid and culture suspensions were prepared as described previously. The minimal inhibition concentration (MIC) of each extract for antibacterial, antifungal and antidermatophytic activity was determined by using broth microdilution techniques as described previously. The microplates were read visually with the aid of an inverted reading mirror after 7 days for dermatophytes. MBC/MFC was defined as the lowest concentration yielding negative subcultures, while any revival of their growth indicates MBS/ MFS of the extract [22-23].

5.3. *In vitro* Antibiofilm Assay

Colorimetric microdilution nearby resazurin microplate assay (REMA), which the turbidity of the mixed aliquots and microorganisms' suspension was measured at 600 nm and 570 nm absorbance was evaluated. IC_{50} values gathered by the spectrophotometric method gave quantitative results. Briefly, the assay performed in bottomed, 96-well plates, which were carried out as serial two-fold dilution of each sample similar with broth microdilution. Growth control wells contain bacteria without the antibiotic; medium nearby drug was included per test and used to calculate percentage inhibition of viability. A stock solution of the resazurin sodium salt powder prepared is used at 0.01% in sterile distilled water and was filtering, thereafter, resazurin (5 µL) added per well, Resazurin dye (7-hydroxy-3H-phenoxazin-3-one-10-oxide) has been used as an indicator of cell viability based on the detection of colorimetric changes caused by the oxidation (blue) and reduction (pink) an indicator for metabolic cell function. The MIC_b was defined by visual examination as the lowest concentration of each samples material that prevented this change in color. The level of reduction was quantified using an enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices VersaMax) at wavelengths of 600 nm and 570 nm, which correspond to the absorption peaks of resazurin and resorufin, respectively. Percentage inhibition of viability was defined as: [(OD samples materials containing wells)-(mean background OD)]/[(OD samples materials free wells)-(mean background OD)]×100, which the samples materials concentration effecting an inhibition of % considered as the inhibition concentration (IC_{50}) [24-25].

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Author contributions: Conception – G.A., K.T., İ.S., O.T., B.Ö.; Design – G.A., K.T., B.Ö.; Supervision – İ.S., B.Ö.; Resources – G.A., İ.S., B.Ö.; Materials – G.A., K.T., İ.S., O.T., B.Ö.; Data Collection and/or Processing – G.A., İ.S., B.Ö.; Analysis and/or Interpretation – G.A., İ.S., B.Ö.; Literature Search – G.A., K.T., B.Ö.; Writing Manuscript – G.A., İ.S., B.Ö.; Critical Review – G.A., B.Ö.

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