Antimicrobial and anti-biofilm activities of *Diospyros lycioides* root, leaf and twig extracts against *Staphylococcus aureus* and *Mycobacterium avium*

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ABSTRACT

This study utilised the minimum inhibitory concentration (MIC) assay and the microtiter plate assay to investigate the antimicrobial and anti-biofilm activities of *D. lycioides* crude methanolic extracts against *Staphylococcus aureus* and *Mycobacterium avium*. Thin layer chromatography was used to qualitatively screen for the presence of phytochemical compounds in the plant methanol and aqueous extracts. Phytochemical analysis revealed the presence of all tested compounds in the methanol extracts, except for saponin. These included coumarin, triterpenoids, anthraquinone, tannins, alkaloids, steroids and flavonoids. On average steroids, tannins, triterpenoids and anthraquinones were the most abundant phytochemicals in both aqueous and methanol extracts, with the highest amounts observed in the methanol extracts. The minimum inhibitory concentration against *M. avium* occurred at $75 mg/ml$, $37.5 mg/ml$ and $18.75 mg/ml$ for the root, leaf and twig methanol extracts, respectively while for *S. aureus* all methanol extracts showed an MIC of $75 mg/ml$. The highest biofilm inhibition against *S. aureus* was due to the methanol extracts of *D. lycioides* leaves (57.2%) and roots (58.1%). However, the twig methanol extracts (57.8%) inhibited *M. avium* biofilm formation the most as compared to the leaf (25.6%) and root (35.3%) extracts. The leaf extracts (40.4%) were moderately active in eradicating *S. aureus* biofilms, whereas for *M. avium* the highest eradication activity was observed with the root extracts (35.6%).

1 Introduction

The problem of infectious diseases has prompted researchers to increase their interest in studying the therapeutic properties of medicinal plants and their natural compounds (Kodali *et al.*, 2013). Many of the microorganisms that are causing infections rely on the formation of biofilms for their survival. Biofilms are communities of cells attached to either a biotic or abiotic surface and enclosed or embedded in a complex extracellular polymeric substance (EPS) (Flemming *et al.*, 2000; Manner *et al.*, 2013). In addition to the EPS, other features such as the presence of flagella and fimbriae, cell hydrophobicity as well as quorum sensing also influence the attachment of microbial cells to the biofilms (Monte *et al.*, 2014; Simões, 2005). Biofilms enhance key features for microbial survival such as allowing microorganisms to trap nutrients, increasing their resistance to antibiotics and overcoming hostile environmental conditions (Khattar *et al.*, 2010; Manner *et al.*, 2013). Biofilm matrices have been shown to prevent the penetration of antimicrobial agents and protecting the microbial cells from the host immune response

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(Ramage *et al.*, 2005; Sauer & Camper, 2001). In addition, biofilms have been associated with various human infections such as otitis media (Hall-Stoodley *et al.*, 2006), chronic wounds (James *et al.*, 2008; Malic *et al.*, 2009), cystic fibrosis (Singh *et al.*, 2000), urinary tract and dental plaque infections (Kodali *et al.*, 2013) as well as AIDS related infections (Barbeau *et al.*, 1998). Moreover, it is emphasized that the presence of biofilms in food processing environments is a potential source of contamination that may lead to food spoilage and disease transmission (Nikolić *et al.*, 2013). The formation of biofilms on medical devices also pose a threat to such medical applications (Manner *et al.*, 2013).

The bacterial species *Staphylococcus aureus* has become increasingly resistant to antibacterial agents and is responsible for various infections including skin and oral infections (Tong *et al.*, 2015). Moreover, *S. aureus* forms biofilms on implanted medical devices such as urinary catheters and intrauterine devices, thus compromising their effectiveness and eventually lead to persistent infections (Costerton *et al.*, 1995; **?**; Otto, 2008). *Mycobacterium avium* on the other hand is a common contaminant (biofilm former) in water reservoirs and an opportunistic pathogen in immunocompromised people such as AIDS infected humans (Barbeau *et al.*, 1998). The current water purification methods are becoming ineffective; thus *M. avium* resistance towards current water purification methods presents a threat to people in poor sanitation. Medicinal plants have traditionally been used to treat various kinds of diseases worldwide (Farnsworth *et al.*, 1985; Ross, 2007; Sofowora, 2005). This is attributed to their secondary metabolites such as phenolics, alkaloids, flavonoids, terpenoids and polyacetylene, volatile sesquiterpenes and monoterpenoids (Nikolić *et al.*, 2013), which interfere with the microbial membrane functions and enzymatic activity (Chanudom *et al.*, 2014). Earlier studies have demonstrated that plant extracts from leaves, fruits, stems and roots are effective in preventing biofilm formation and adherence (Nikolić *et al.*, 2013). Some plant extracts have anti-quorum sensing properties which are essential in combating microbial biofilm formation (Musthafa *et al.*, 2013).

Dyospyros lycioides is a medicinal plant commonly found in the Northern part of Namibia, mostly in Oshikoto, Ohangwena and Omusati regions, and has various local names including Oshiyugulu, Oshimumu and/or Oshilagula. It is commonly recognised by its ability to cause an orange-yellow colour formation in the mouth upon chewing, which was believed to have been one of the ways to attract mates among the human species in the old ages. This plant has traditionally been used as a mouth cleansing agent as it is known to effectively destroy plaques. In support of this, other studies reported that the twigs of *D. lycioides* have been shown to inhibit growth of certain oral pathogens in vitro and are thus used frequently by rural and urban people in Namibia as toothpaste (Cai *et al.*, 2000). Furthermore, many chewing sticks are also commonly used as mechanical oral hygiene aid in the African and Middle Eastern countries (Li *et al.*, 1998). Hence, the mouth cleansing properties of this medicinal plant could mean that the same plant may be able to destroy all other microorganisms relying on biofilms formation for their survival.

2 Materials and methods

2.1 Plant materials collection and processing

Plant materials of *D. lycioides* used in this study were collected from the Northern part of Namibia, in Oshikoto region (Oniipa constituency, Ondonga district, Oshali village) in 2016. The confirmation of the plant was done by enquiring with local people who have been using this plant as a mouth cleansing agent. Plant materials were rinsed with tap water to remove sand and then separated into root, leaf and twig parts. The twigs and roots were cut into small pieces and all plant parts were stored to dry at room temperature for 4 weeks. A voucher specimen of the collected plant was submitted to the National Botanical Research Institute for identification. The root, leaf and twig materials of *D. lycioides* were then crushed into powder using a laboratory blender. The crushed materials were placed in separate Ziploc bags, sealed and stored in the fridge at $4^{\circ}C$ until use.

2.2 Preparation of organic and aqueous plant extracts

For the organic extracts, $10\,g$ of the crushed root, leaf and twig materials were added to separate conical flasks with $100\,ml$ of absolute methanol each. After mixing, the flasks were sealed with parafilm and incubated at room temperature in a cupboard for 48 hours. The solutions were filtered, and the filtrate concentrated using a rotary evaporator at the pressure of 40 mbar and a temperature of $40°C$. After evaporation, the extracts were freeze dried into powder for four consecutive days. The dry extracts were scraped off, and the yield was recorded in grams. The dry extracts were placed in separate Ziploc bags and stored at $-20°C$.

For the aqueous extracts, $10\,g$ of root, leaf and twig crushed materials were weighed and placed in separate conical flasks labelled accordingly. Hundred millilitres of distilled water was added to each of the flasks containing the materials and mixed thoroughly by swirling the flasks. Each of these solutions was transferred into $50\,ml$ centrifuge tubes and then heated in a water bath at $60°C$ for 2 hours and at the same time being vortexed after every 30 minutes. After the 2 hours' period, the solutions were centrifuged for 10 minutes at 1500 rotations per minute (rpm). Following this was filtration of the solution using Whatman number 1 filter paper and the filtrates were collected. The filtrates were concentrated using a rotary evaporator at 5 mbar and a temperature of $70°C$. After evaporation, the extracts were then freeze dried into powder for four consecutive days. The dry extracts were scraped off, and the yield was recorded in grams. The dry extracts were placed in separate Ziploc bags and stored at $-20°C$.

2.3 Phytochemical analysis

Thin Layer Chromatography (TLC) was used to screen for the presence or absence of the phytochemical compounds listed in Table 1. TLC plates were spotted with solutions of the three different organic and aqueous plant extracts. The solvent systems, chromogenic reagents, controls and expected results for each compound analysed are depicted in Table 1 (Harborne, 1998).

Table 1: An outline of how the phytochemical analysis was carried out using TLC method

Phytochemical compound	Chromogenic reagent	Solvent systems	Positive control	Expected results
Coumarin	1.3g copper sulphate+$17.3\,g$ sodium citrate+$10\,g$ anhydrous sodium carbonate+$200\,ml$ water	Chloroform	Coumarin	Fluorescence in long wavelength UV- Blue
Triterpenoids	Liebermann reagent: cool $5\,ml$ acetic acid and $5\,ml$ H2SO4 mix with cooling, mix with $50\,ml$ ethanol.	Hexane: Ethyl acetate (17:3)	B-sitosterol	Blue, green, yellow
Saponin	Vanillin +ethanol+ sulfuric acid. $15\,g$ vanillin in $250\,ml$ ethanol and $2.5\,ml$ H2SO4.	Chloroform: methanol: water (7:3:1)	Saponin	Blue fluorescence without spray
Anthraquinone	10% potassium hydroxide in methanol ($0.1\,g$ in $200\,ml$).	Ethyl acetate: methanol: water (100:17:13)	Alixaria	Blue, yellow, brown
Tannins	1% ferric chloride in 50% aqueous methanol	1% potassium hydroxide in methanol	B-sitosterol	Purple, green
Alkaloids	Dragendorff reagent: A) 0.85 basic bismuth citrate+$10\,ml$ acetic acid+$40\,ml$ water. B) $8\,g$ potassium hydroxide in $30\,ml$ water. Mix A+B (1:1) and dilute in acetic acid and water (1:2:10).	Chloroform: ethanol (9:1)	Quinine	Orange
Steroids	Phosphoric acid+ water (1:1) (A). 15% methanol in phosphoric acid, heat for 15-20minutes at $120°C$	Chloroform+ acetone (90:10)	B-sitosterol	Fluorescence in long UV light, dark spots upon heating at $120°C$ for 15-20 minutes.
Flavonoids	Antimony chloride: 10% antimony chloride in chloroform ($0.2\,g$ in $200\,ml$).	Butanol: acetic acid: water (4:1:5)	Quercetin	Fluorescence spots at 360nm. Yellow, orange, blue

2.4 Bacterial strains used

The swabs of *Staphylococcus aureus* and *Mycobacterium avium* were provided by the Biological Sciences Department of the University of Namibia. Overnight pure cultures of these bacteria were grown in nutrient broth and nutrient agar media at $37°C$.

2.5 Antimicrobial assay

This procedure was carried out using the broth dilution method as described in previous studies (Boulaaba *et al.*, 2015). Overnight bacterial cultures were diluted 1:100 and the optical densities (OD) were measured with a UV spectrophotometer at 600nm. These cultures were adjusted to McFarland standards in the range $0.1-0.5$. The turbidity of each bacterial culture was adjusted to the desired range by dilution if OD values were higher

than the standards or otherwise added from the concentrated culture if OD values were less than the standards (Khanam *et al.*, 2015). Ten ($10\,\mu l$) of the cultures were then inoculated with double fold dilutions of plant extracts ranging from $1.17\,mg/ml$ to $300\,mg/ml$ and incubated at $37°C$ for 24 hours. After incubation, each test tube's content was compared to the controls to determine the lowest concentration with no microbial activity (MIC). Furthermore, $2\mu l$ of content from the MIC test tubes with no growth was spread onto nutrient agar plates and incubated for 24 hours at $37°C$ to confirm if there was no microbial growth. The controls to which the test tubes were compared after incubation included; culture diluted in broth, each extract diluted in broth, as well as broth alone. The antibiotics Vancomycin ($0.03\,mg/ml$) and Tetracycline ($0.03\,mg/ml$) were used as positive controls.

2.6 Antibiofilm assay

2.6.1 Biofilm formation and inhibition

This procedure was carried out according to previously published methods (Christensen *et al.*, 1985; do Monte, 2013; Merritt *et al.*, 2011) with some modifications. Cultures were inoculated into $50\,ml$ falcon tubes containing $5\,ml$ brain heart infusion broth and grown to stationary phase at $37°C$ for 24 hours. The cultures were diluted 1:100, and $100\,\mu l$ of diluted culture was added to each of the six wells of a sterile round-bottom 96-well microtiter plate. Three of the six wells were inoculated with 10µl of $4.68\,mg/ml$ of each plant extract which was just below the pre-determined minimal inhibitory concentration (MIC). Sixteen wells contained $200\,\mu l$ sterile broth alone as controls. The plates were parafilmed to prevent them from drying out and incubated at $37°C$ for 24 hours.

Planktonic cells were removed after incubation by placing the microtiter plate upside down on a thick piece of towel paper and gently tapped thus allowing the paper to soak up the cultures. In addition, each well was washed once by pipetting $400\mu l$ double distilled water into it and inverting the plate onto a towel paper to remove the remaining planktonic cells. The biofilms in the wells were dried and fixed in an incubator at $60°C$ for 45 minutes.

The wells were stained with $125\mu l$ of 0.1% crystal violet, incubated for 15 minutes at room temperature and the crystal violet was discarded. Excess stain was removed by washing $5\times$ with $400\,\mu l$ double distilled water and the plates were then air dried in a fume hood for 15 minutes. The wells were de-stained with $200\,\mu l$ of 30% glacial acetic acid and $125\,\mu l$ of the content of each well was transferred to a new 96-well microtiter plate labelled accordingly. Absorbance of stained biofilms was determined with a plate reader at 595 nm. The readings of the sterile brain heart infusion control wells were averaged and then subtracted from the test readings. The resulting differences were averaged and used to calculate the standard deviations and standard errors. The classification method by Christensen *et al.* (1985) for interpreting biofilm formation results was used in this study.

The equation $\mathrm{I}\% = [1 - (A_{595}\text{ of test}/A_{595}\text{ of non-treated control}) \times 100]$, was used to calculate percentage inhibition (Kawsud *et al.*, 2014; Teanpaisan *et al.*, 2014). The criteria for antimicrobial activity used in this study are: Highly active: $> 85\%$ inhibition, moderately active: $> 40\%$ inhibition and inactive: $< 40\%$ inhibition (Manner *et al.*, 2013).

2.6.2 Biofilm formation and eradication

The same procedure was followed as for the inhibition assay, except that inoculation with $10\,\mu l$ of $4.68\,mg/ml$ of plant extracts was performed after incubation of cultures to stationary phase and removal of planktonic cells. The plate was then further incubated overnight at room temperature. The wells were also stained with 0.1% crystal violet and destained with 30% glacial acetic acid and the absorbance was read at 595 nm. The percentage eradication was calculated using the equation $\mathrm{E}\% = [1 - (A_{595}\text{ of test}/A_{595}\text{ of non-treated control}) \times 100]$ (Kawsud *et al.*, 2014; Teanpaisan *et al.*, 2014).

3 Results

3.1 Yields of *D. lycioides* extracts

Overall, methanol (organic) extracts showed higher percentage yields compared to aqueous extracts. For methanol extracts, the leaves, roots and twigs of *D. lycioides* yielded 21%, 11% and 11% respectively. The percentage yield for the leaf, root and twig aqueous extracts were 7%, 9% and 6%, respectively.

3.2 Phytochemical screening of the root, leaf and twig extracts of *D. lycioides*

Root, twig and leaf extracts of *D. lycioides* were analysed qualitatively to detect the presence of different phytochemical compounds (Table 2). Most compounds were detected in the methanol extracts. The highest amounts of steroids, alkaloids, coumarins and tannins were present in the methanol extracts as compared to aqueous extracts. On the contrary, only aqueous root extracts showed the presence of saponin among all extracts. Moreover, anthroquinones were equally present in both methanol and aqueous extracts.

Table 2: Phytochemical analysis for methanol and aqueous extracts of different parts of *D. lycioides*

		Flavonoids	**Alkaloids**	**Saponin**	**Steroid**	**Coumarin**	**Tannins**	**Triterpenoids**	**Anthraquinones**
Methanol extracts	Root	+	+	-	+++	+	+++	+	++
	Leaf	+	+	-	+++	+	++	++	+++
	Twig	++	+	-	+++	++	++	+	+
Aqueous extracts	Root	+	-	+	+	-	-	++	+++
	Leaf	+	-	-	+	-	+	++	++
	Twig	-	++	-	+	-	+	+	+

Key: +++ For **highly** present; ++ for **moderately** present; + for **low** presence and – for **absence** of specific phytochemical compounds

3.3 Antimicrobial assay of *D. lycioides* root, leaf and twig extracts

The minimum inhibitory concentration was determined only for the methanol extracts as they showed greater antimicrobial activity in the disc diffusion assay (data not shown). The MIC against *M. avium* occurred at $75 mg/ml$, $37.5 mg/ml$ and $18.75 mg/ml$ for the root, leaf and twig methanol extracts, respectively while for *S. aureus* all methanol extracts showed an MIC of $75 mg/ml$.

3.4 Anti-biofilm activity of *D. lycioides* root, leaf and twig extracts

The leaf and root methanol extracts exhibited high biofilm inhibition against *S. aureus*, whereas the twig methanol extracts showed the highest level of inhibition against *M. avium* (Figure 1A). Interestingly, Fig. 1A also shows that the twig methanol extracts of *D. lycioides* were unable to inhibit the biofilm formation of *S. aureus*.

Except for the leaf methanol extracts, the root and twig methanol extracts of *D. lycioides* were less effective in eradicating preformed biofilms of *S. aureus*, while the root methanol extract was more effective in eradicating preformed biofilm of *M. avium* (Fig. 1B). In comparison to the biofilm inhibition capabilities (Fig. 1A), most extracts were relatively inactive in eradicating preformed biofilms in both *S. aureus* and *M. avium* (Fig. 1B).

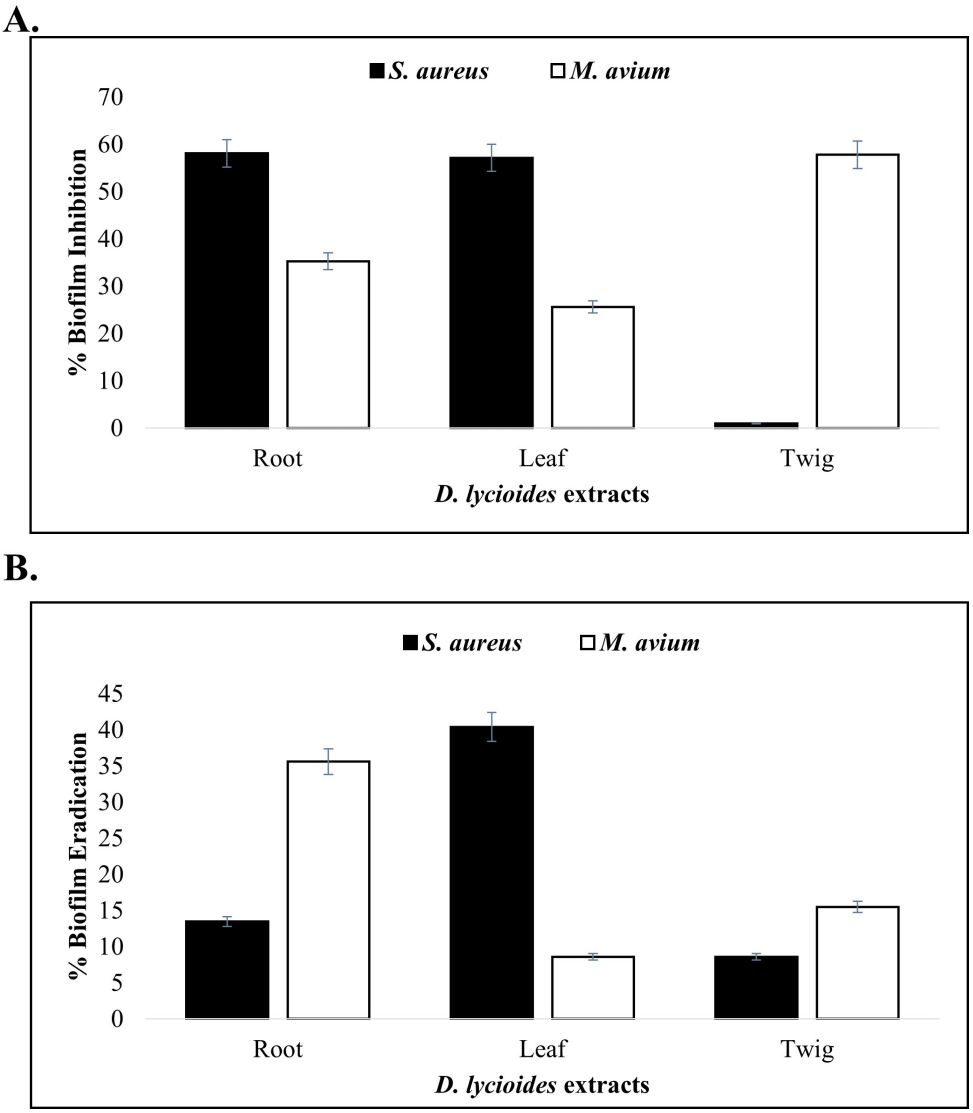

Figure 1: Antibiofilm activity of *D. lycioides* root, leaf and twig methanol extracts against *S. aureus* and *M. avium*. **A.** depicts the percentage biofilm inhibition, and **B.** depicts the percentage biofilm eradication calculated using the formula $\%\text{I/E} = [1 - (A_{595} \text{ of test}/A_{595} \text{ of non-treated control}) \times 100]$ (Kawsud *et al.*, 2014; Teanpaisan *et al.*, 2014).

4 Discussion

In line with earlier reports (Akintobi *et al.*, 2011; Akujobi, 1997; Bowyer *et al.*, 2015; Chigayo *et al.*, 2016; Iloki-Assanga *et al.*, 2015), this study provided additional evidence to the effectiveness of using methanol for extraction of plants bioactive compounds.

According to the MIC results, *M. avium* was found to be highly susceptible to all the methanol extracts of *D. lycioides* tested in this study, as compared to *S. aureus*. This was strongly validated by the fact that the twig extracts were able to show activity against *M. avium* at a concentration as low as $18.5 mg/ml$. The twig

extracts among others were found to contain flavonoids and alkaloids and these are known to exhibit inhibitory ability against microbial DNA ligase, cytoplasmic membrane functions as well as energy metabolism (Cushnie & Lamb, 2005). Hence, the observed antimicrobial results against *M. avium* could be due to the content of the twig extracts as shown in this study.

Meanwhile, the positive controls Vancomycin and Tetracycline for *S. aureus* and *M. avium*, respectively demonstrated the highest activity at a concentration as low as $0.03mg/ml$, in comparison to the extracts. This observation is no surprise, given that many other studies have reported antibacterial activity at the MIC of *D. lycioides* of as low as $0.15mg/ml$ (Li *et al.*, 1998; Mbanga & Magumura, 2013), which is close to the concentration of the positive controls used here.

Notably, high anti-biofilm activity was observed against both bacteria, with *S. aureus* being more susceptible to the leaf and root extracts than the twig extracts. This may be associated with the high presence of anthraquinones which were detected in the root and leaf methanol extracts. In support of this, anthraquinones have previously been linked with anti-biofilm formation and anti-haemolytic activity of *S. aureus* (Lee *et al.*, 2016).

Contrastingly, a high percentage of *M. avium* biofilm inhibition was observed due to the twig methanol extracts. The high presence of triterpenoids in the twigs extracts may be contributing to this observed anti-biofilm activity. The terpenoid family is reported to contain anti-mycobacterial activity due to their lipophilic nature and thus ability to penetrate the mycobacterial membrane (Bunalema, 2010). It is further emphasized that the crude methanol twig extracts of the Namibian chewing stick inhibit the growth of certain gram-positive bacteria (Cai *et al.*, 2000). Furthermore, the extracts of this plant have also been shown to exhibit microbial activities against certain other bacteria such as *Streptococcus mutans* and *Porphyromonas gingivalis* (Li *et al.*, 1998; Mbanga & Magumura, 2013).

On the other hand, anthraquinones and triterpenoids could be responsible for the biofilm eradication capacity of the leaf methanol extracts against *S. aureus*. These phenolic compounds have been shown previously to be effective against *S. aureus* (Hatano *et al.*, 2005, 1999). It is further noted that anthraquinones and tannins are known to exhibit several antibacterial activities (Manojlovic *et al.*, 2000), and this may be the reason for the eradication activity of the root and leaf methanol extracts observed in this study.

5 Conclusion

The current study demonstrated high potential for using *D. lycioides* as an agent in treating skin infections, water contamination and other problems associated with *S. aureus* and *M. avium*. The phytochemical compounds of *D. lycioides* in the methanol extracts show remarkable antimicrobial as well as anti-biofilm activities against *S. aureus* and *M. avium*. This antimicrobial capacity denotes that the plant is a potential source of food and beverage preservatives, as well as a source of water decontamination agents. Therefore, further research on this shrub may promote the discovery and development of novel antimicrobial agents.

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