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WORD COUNT 4037

TIME SUBMITTED 28-AUG-2023 07:27PM

PAPER ID 102317604

Phytochemical Analysis and antibacterial activity of W*ashingtonia filifera* (Lindl.) H. Wendl. fruit extract from Saudi Arabia

Abstract:

This work aimed to assess the antimicrobial potential of *Washingtonia filifera* extracts on some human pathogens. Agar well diffusion and minimum inhibitory concentrations (MIC) methods have been used to assess the antimicrobial activities of *W. filifera* extract against *Staphylococcus aureus, Klebsiella pneumonia, Acinetobacter baumannii, Escherichia coli, and Candida albicans*. Only the ethyl acetate (ETAC) and methanol extracts revealed antimicrobial activity against tested microorganisms. *S. aureus* appears to be the most sensitive microbes to the ETAC extract with equal inhibition zone (30 mm) and MIC (65 μg/mL) values. This is followed by *K. pneumoniae*, *E. coli*, *and A. baumanni*, respectively. The plant extract had different phytochemical constituents such as alkaloids, sterols, and polyphenols. Column chromatography of the ETAC extract resulted in the loss of inhibitory effect at the highest concentration tested (50 mg/mL) against tested microorganisms. The haemolytic activity of the different extracts was found in the following order: Hexane (83.57%) > ETAC (35.71%) > chloroform (23.57143) > methanol (0.71%) based on the highest concentration tested (8.3 mg/mL). In conclusion, ETAC extract was the most promsing extract among extracts tested. Secondary plant metabolites are of great value as natural antimicrobial agents.

Keywords: fan palm, antimicrobial activity, blood hemolysis, secondary metabolites

1. Introduction

The emergence of multidrug-resistant microorganisms has negatively impacted the global effectiveness of antibiotics (D'Andrea et al., 2019; Falcone and Paterson, 2016; Algammal et al., 2023). As a result, it increases healthcare costs, mortality, and morbidity (Opperman and Nguyen, 2015). The condition is further worrying by the lack of efficient laboratory diagnostics, access to suitable antimicrobials, and surveillance systems in low-income countries. If there were no serious efforts to look for new antimicrobial agents, the health care costs, mortality, and morbidity would rise (Kebede et al., 2021; Morehead and Scarbrough, 2018; Raoult and Paul, 2016). To this effect, the search for novel antimicrobial agents from botanical sources to overcome the health and socio-economic burden caused by multidrug-resistant pathogens is necessary (Bakal et al., 2017; Solomon and Oliver, 2014; Aldhanhani et al., 2022)

Plants were used centuries ago to treat various conditions in different civilizations. Around 80% of the world's population are dependent on natural remedies to treat various ailments (Oyebode et al., 2016), and about 75% of approved drugs are isolated from natural sources (Cragg and Newman, 2013). Medicinal plants contain polypeptides, essential oils, tannins, terpenoids, alkaloids, polyphenols, flavonoids, and coumarins (Chandra et al., 2017; Aldhanhani et al., 2022). These secondary metabolites are used as a source for discovering antibiotics and treating various diseases (Cragg and Newman, 2013). The extract of *Polysphaeria aethiopica, Euphorbia depauperata, Cirsium englerianum, Lippia adoensis, Cucumis pustulatus, Discopodium penninervium, and Rumex abyssinicus* have antimicrobial

activities against resistance and nonresistance microbes such as *E. coli, S. aureus*, *K. pneumoniae*, *Streptococcus pyogenes*, *Trichophyton mentagrophytes* , and Candida *albicans* (Kebede et al., 2021). In another study, the dichloromethane and ETAC extract of *W. somnifera* was active against Methicillin-resistant *Staphylococcus aureus* (MRSA), *T. mentagrophytes*, and *Microsporum gypseum*, but not active against *C. albicans* and *Cryptococcus neoformans* (Mwitari et al., 2013). The extracts of *Thevetia peruviana*, *Erythrophleum suaveolens*, and *Euphorbia hirta*, reported to possess antibacterial effects against *E. coli, Pseudomonas, K. pneumonia, MRSA, Salmonella* spp. and *Proteus* spp. (Niranjan et al., 2017; Sharifi-Rad et al., 2016).

It has been pointed out that natural products are significant sources of new bioactive compounds (Dar et al., 2017; Khalil et al., 2022). Botanical sources are valuable for novel bioactive secondary metabolites due to their ecological diversity and diverse chemical constituents (Kenneth-Obosi and Babayemi, 2017; Xylia et al., 2022).

W. filifera (family of Arecaceae) (Fig.1) is commonly known as desert fan palm. *W. filifera* is the native palm of the Western United States (Uluçınar, 2017) and has been cultivated in the Mediterranean and elsewhere (El-Sayed et al., 2006) 2006). The fruits are creamy white, oval in shape, and around 13 cm in size (Watson, 1994). When they mature, their color changes to black, and the seeds (8 mm) are present inside the part of the fruits (Uluçınar, 2017). Yet, there are limited details of *W. filifera* antimicrobial potential and toxicity as therapeutic agents for standard and clinical microbes. Therefore, the present study aims to assess the antimicrobial activity, toxicity and phytochemical analysis of *W. filifera* fruit extracts using different solvents.

2. Materials and methods

2.1. Collection and authentication of *W. filifera*

The unripen fruits of *W. filifera* (Figure 1) were collected from the King Saud University campus in September 2021, Riyadh, Saudi Arabia. A taxonomist identified the plant at the Department of Botany and Microbiology, King Saud University. The specimen (Voucher number BRC-040) was deposited in herbaria for future reference.

2.2. Preparation of extract

2.2.1. Soxhlet extraction

Sixty grams of the coarsely powdered fruit of *W. filifera* was Soxhlet extracted by sequential extraction in solvents of increasing polarity (hexane, chloroform, ETAC, and methanol). The extraction was carried out for 12 hrs or until colorless and then concentrated by Rotavapor at 45 °C.

2.2.2. Silica Gel Column Chromatography.

Five grams of the ETAC extract were dissolved in ETAC, mixed with silica gel, and left until completely dried (1 mL). This mixture was loaded on a chromatographic glass column (4 cm, 30 cm height) packed with chloroform slurry of silica gel 60 silanized (Merk, Germany) previously activated (100 °C for 1 h). The column was eluted with toluene:

chloroform (70:30), toluene: chloroform: methanol (7:2:1), toluene: chloroform: methanol (6:2:2), chloroform: methanol (3:7), and methanol (100%). Five fractions (400mL each) were collected. Each fraction was evaporated by Rota vapor at 45 °C, and the stock solution was prepared (10 mg/mL).

2.3. Phytochemical screening

Phytochemical analysis was conducted on extracts and fractions derived from *W. filifera*. Identification tannin/phenol was assessed using Ferric Chloride´s test, alkaloids using Dragendorff´s test, saponins by foam appearance, steroids/triterpenoids using Liebermann-Buchard´s test, sugars using throne´s reagent, flavonoid by Shinoda´s test (HUI et al., 2018).

2.4. Test microorganisms

Clinical and standard isolates, including *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Klebsiella pneumonia* ATCC (BAA-1705), *Acinetobacter baumannii* ATCC BAA-747, *Staphylococcus aureus* (clinical isolate), *Acinetobacter baumannii* (clinical isolate), and *Candida albicans* ATCC-66027 were collected from Microbiology Laboratory, King Saud University, Riyadh.

2.5. Inoculum preparation

All microbes were refreshed in Petri dishes containing nutrient agar or potato dextrose agar by incubation for 20 hours at 37°C. A loopful of grown bacteria was added to 5 ml of broth

culture. The absorbance was adjusted at 600 nm and diluted to attain a cell count of 10^7 CFU/ml using a spectrophotometer (Obeidat et al., 2012).

2.6. Agar well diffusion assay

Agar well diffusion method was carried out as previously reported (Abutaha et al., 2021). The broth culture that was standardized in the preceding section was evenly applied onto Petri plates using a sterile cotton swab. Six milimeter wells were made with a cork borer. Twenty microliters of 10 mg/ml of each extract was pipetted into each well, making the final concentration 200 μg/well. DMSO was used as a negative control. The plates were kept for about 2 h at 25 °C. The plates were incubated for 24 h at 37 °C. A ruler was used to measure the inhibition zone. Each assay was carried out in three independent replicates, and the mean was calculated.

2.7. Determination of the Minimum Inhibitory Concentration (MIC)

The promising extracts were serially diluted using 96 well-plates (NEST, China). A volume of 100 µL of MHB was added to each well. The columns (A to E) were loaded with 50 µl stock solution (10 mg/ml) of the extract except for the last three rows in which equal amount of DMSO (F row), standard antibiotic (G row), and sterility control (50 µl of MHB) (H row) were added. A series of three-fold dilutions were carried out. Subsequently, bacterial suspension (100 µl) was added to the wells, with the exception of the 7th and 8th rows, which were designated for sterility control and color contrast control, respectively. Following the serial dilution, a resulting concentration range of 1.6 mg/mL to 0.0008 mg/mL was achieved. Ultimately, the plates were placed in an incubator at 37°C for a duration of 24 hours. The

MIC value is determined as the minimum extract concentration where no turbidity was observed. All experiments were conducted in triplicate.

2.8. Hemolytic activity (HA)

The Hemolytic activity of all the extracts was investigated using human erythrocytes (hRBCs) following the method of a previous report (Abutaha et al., 2021). A 5% (v/v) suspension of erythrocytes was mixed with different extract concentrations in a 96-well plate at 37°C for 30 min. Plates were pelleted by centrifuged for 3 min at 3,000 rpm. The supernatant was transferred to 96-well and used to calculate the released hemoglobin at 540 nm (ChroMate, England). Phosphate buffer saline was employed as the negative control, while Triton X-100 (1%) served as the positive control. Three separate experiments were conducted in duplicate, and the hemolysis percentage was calculated using the following equation.

$$\text{Hemolysis Percentage} = \frac{\text{Absorbance of sample}}{\text{The absorbance of positive control}}$$

2.9. Statistical analysis

The results are expressed as mean ± standard deviation. The statistical analyses were performed using Microsoft Excel, and the graphs were generated using OriginPro 8.5 software.

142 **3. Result**

143 The tested plant extracts showed a variation in the percentage of yield. The methanol extract
144 showed the highest yield at 6%, whereas the hexane extract displayed the lowest yield of
145 1.38% (Figure 2). Chromatographic separation of ETAC extract was carried out and
146 produced a total of 5 fractions. F3 and F2 fractions exhibited the highest yields at 40% and
147 35%, respectively. Conversely, the lowest yields were identified in F4 and F5 fractions at
148 3.2% and 2.5%, respectively. The phytochemical investigation of the separated fractions
149 (Table 1) showed the presence of polyphenols and alkaloids in F3 and F4 fractions (Table 1).
150 F2 and F3 gave only a faint coloration in alkaloid reactions. The remaining fractions failed
151 to show the presence of any of these secondary metabolites tested. No antioxidant activity
152 was observed in all the solvents and fractions obtained.

153 This work aims to assess the antimicrobial potential of hexane, chloroform, ETAC, and
154 methanol extracts of W. *filifera* on some human pathogenic microorganisms. Agar well
155 diffusion and minimum inhibitory concertation methods have been used to assess the
156 antimicrobial potential of W. *filifera* extracts against *S. aureus* (Gram-positive bacteria), *K.*
157 *pneumoniae, E. coli, and A. baumanni* (Gram-negative bacteria), and one fungus (*C.*
158 *albicans*). Only the ETAC and methanol extracts exhibited antimicrobial activities against
159 tested microorganisms. ETAC and methanol extracts showed antibacterial activity against
160 tested bacterial strains and *C. albicans*. However, the ETAC extract was more effective
161 against all tested organisms. *S. aureus* (ATCC-29213) and *S. aureus* (clinical) appear to be
162 the most sensitive microbes to the ETAC extract with equal inhibition zone (30 μg/mL) and

163 MIC (65 μg/mL) values. This is followed by *K. pneumoniae, E. coli, A. baumanni* (Clinical)

164 and *A. baumanni* (ATCC - BAA-747) respectively (Table 2).

165 The haemolytic activity of the different solvent extracts of *W. filifera* fruit was screened

166 against normal hRBCs. Haemolytic is reported as a percentage hemolysis of three

167 experiments. ETAC and chloroform extracts exhibited very low haemolytic effects toward

168 human erythrocytes, whereas hexane extract showed the maximum haemolytic activity and

169 ranked first in the list. However, methanol extract showed no haemolytic activity towards

170 normal hRBCs. These extracts showed an increase in haemolytic activity with the increasing

171 concentration of the extracts (Figure 3). The EC_{50} for chloroform, ETAC, and methanol

172 extracts were not calculated because the hemolysis of the highest tested concentration (8.3

173 mg/mL) was less than 40%. Based on the highest concentration tested (8.3 mg/mL), the

174 haemolytic activity of the different extracts was found in the following order: Hexane

175 (83.57%) > ETAC (35.71%) > chloroform (23.57143) > methanol (0.71%). The IC_{50} value

176 was calculated only for hexane extract (EC_{50}:280 μg/mL); the hexane extract showed the

177 maximum hemolytic activity and ranked first in the list.

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4. Discussion

WHO has recognized the rise of microbial antibiotic resistance as a global health risk that necessitates the attention of countries and organizations (van Duin and Doi, 2017). Therefore, there is a dire need to search for new antimicrobial agents from natural resources to overcome the rising threat of resistant pathogens. This study revealed that ETAC and methanol extracts had broad-spectrum antimicrobial activity. *S. aureus* (gram-positive) was the most susceptible bacteria of all tested pathogens. This finding agrees with several reports that gram-positive bacteria are more susceptible to botanical extracts than gram-negative ones (*K. pneumonia*, *E. coli*, and *A. baumannii*).

A. baumannii is a significant and challenging pathogen that has become a global concern. It causes a wide range of infections, particularly in immunocompromised individuals within intensive care units. A significant concern linked to this pathogen is its capacity to develop resistance to a vast majority of antibiotics that are employed in clinical practice (Elwakil et al., 2023).

The outbreaks of *A. baumannii* infections have been reported globally. At present, antibiotic choices to treat *A. baumannii* are limited due to multidrug-resistant (Perez et al., 2007), and current antimicrobial agents in the pharmaceutical pipeline do not appear promising (Karageorgopoulos and Falagas, 2008). To our knowledge, this is the first report of anti-A. baumannii activities of *W. filifera* extract, although weak inhibitory activities of *W. filifera* against other pathogenic bacteria, have been reported. For example, 70% methanol and ETAC extracts of mature and immature seeds were shown to inhibit *S. aureus* and *E. coli* (Uluçınar, 2017). This weak activity reported could be due to the methods of extraction

201 adopted. On the other hand, other biological activities have also been reported. The alcoholic
202 seed extracts of *W. filifera* showed inhibitory activity to xanthine oxidase, α-glucosidase,
203 butyrylcholinesterase, α-amylase, elastase, and collagenase (Floris, 2021).

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204 The mechanical stability of the hRBCs membrane is an excellent indicator to assess in vitro
205 effects of secondary metabolites when screening for cytotoxicity (Baillie et al., 2009; Sharma
206 and Sharma, 2001). Treating cells with toxic secondary metabolites may cause loss of
207 membrane lipid bilayer integrity and death of cells due to cell lysis (Tiwari et al., 2011). The
208 hemolytic activity of the *W. filifera* extracts (ETAC and methanol) gave a much higher range
209 than that of the MIC values against all microorganisms tested.

210 In the present study, different bioactive compounds (alkaloids and polyphenolic compounds)
211 extracted from *W. filifera* inhibited the growth of both clinical and reference isolates. Other
212 reports have also documented that the plant extracts containing polyacetylenes, tannins,
213 terpenoids, alkaloids, coumarins, polyphenols, and flavonoids which are promising
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214 antimicrobials against different human pathogens (Dholaria and Desai, 2018; Habtamu et al.,
215 2010; Keita et al., 2022). This inhibitory effect of phytocompounds come from the
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216 disintegration of the outer membrane, disruption of the biochemical pathway, and inhibition
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217 of protein synthesis (Ellington et al., 2010; Shriram et al., 2018). Therefore, a lot has to be
218 carried out to investigate the antimicrobial potential of plant extracts to treat human-resistant
219 pathogens. On further fractionation, column chromatography of the ETAC extract resulted
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220 in the loss of inhibitory effect at the highest concentration tested (50 mg/mL) against all
221 tested microorganisms. We hypothesized that the antimicrobial activity of *W. filifera* extract
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222 might have acted synergistically or additively to produce the activity observed with the parent

223 fraction. This result is in agreement with the previously published papers (Nwodo et al.,
224 2010).

225 ## 5. Conclusion

226 The study revealed that the use of methanol and ETAC solvents in extracting *W. filifera*
227 resulted in antibacterial activity, surpassing the effects of n-hexane and chloroform solvents.
228 Notably, the ETAC extract exhibited potent antibacterial activity. Among the tested
229 microorganisms, *S. aureus* demonstrated the highest sensitivity to the ETAC extract, showing
230 equal inhibition zone (30 mm) and MIC (65 μg/mL) values. The ETAC and chloroform
231 extracts displayed minimal toxicity towards human erythrocytes, while the hexane extract
232 exhibited the highest level of haemolytic activity (EC50: 280 μg/mL). Further investigations
233 on toxicity are needed to uncover the potential of W. filifera extract as an effective
234 antibacterial agent.

235 **Acknowledgement**

236 Researchers Supporting Project number (RSPD2023R757), King Saud University, Riyadh,
237 Saudi Arabia.

238 **Conflicts of interest**

239 The authors declare no conflict of interest.

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Table 1: Preliminary phytochemical analysis of different solvent extracts and fractions of *Washingtonia filifera* fruits

Phytochemical Tests	Hexane	Chloroform	Ethyl acetate	Methanol	F1	F2	F3	F4	F5
Polyhenol	+	+	++	+	-	-	-	+++	+++
Alkaloid	-	-	+	+	-		+	++	+
Tannin	-	-	-	-	-	-	-	-	-
Sterols	-	-	-	+	-	-	-	-	-
Anthraquinone glycosides	-	-	-	-	-	-	-	-	-
Antioxidant	-	-	-	-	-	-	-	-	-

364 **Table 2.** Antimicrobial potential of the *Washingtonia filifera* ethyl acetate and methanol extracts.

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Microorganism	Origin	Resistance phenotype	Well assay zone (mm)	MIC (μg/mL)	Well assay zone (mm)	MIC (μg/mL)
			Ethyl acetate extract		Methanol extract	
Microbes						
K. pneumoniae	ATCC (BAA-1705)	S	20 ±0.01	26	15±0.6	520
E. coli	ATCC - 25922	S	20±0.09	26	17±0.6	520
A. baumanni	ATCC - BAA-747	S	15±0.6	26	10±0.6	520
A. baumanni	Clinical	Amox/K, clav, amx, ampicillin , cxm,fix, cefpodoxime	16±1.0	26	10±1.0	520
S. aureus	Clinical	S	30±0.6	6.5	30±0.6	65
S. aureus	ATCC-29213	S	30±0.6	6.5	25±0.01	65
C.albican	ATCC-66027	S	8±1.2	200	8±0.9	200

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368 The values are expressed as mean ±SD of the three replicates

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386 **Figure 1 :** *Washingtonia filifera* tree (left) and its fruits (right) cultivated in the Kingdom of Saudi Arabia,
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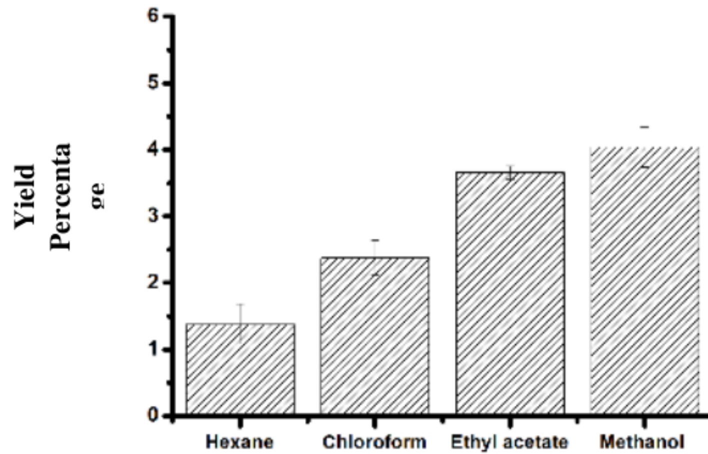

399 **Figure 2:** Yield obtained from extracting *Washingtonia filifera* in solvents of increasing polarity using
400 soxhlet extractor. The values are expressed as mean ±SD of the three replicates

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405

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mg/mL

Hemolysis percentage

Figure 3: Haemolytic activity was examined after incubation at 37°C for 30 min using different solvent extracts of *Washingtonia filifera* fruits against human erythrocytes. The values are expressed as mean ±SD of the three replicates.

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