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Effects of Solvents on the Antimicrobial Activities of Crude Extracts Produced from *Conocarpus* species Leaves

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***Abstract*— This study aimed to determine the most effective solvent for extracting antimicrobial compounds from powdered leaves of *Conocarpus* species. The powdered leaves were extracted separately using petroleum ether, aqueous methanol (80%), and chloroform. The antimicrobial activity of each crude extract was tested against several pathogenic microorganisms at various concentrations. The aqueous methanol extract demonstrated the strongest antimicrobial activity against *Candida albicans*, *Staphylococcus aureus*, *Streptococcus mutans*, and *Escherichia coli* compared to the petroleum ether and chloroform extracts. The minimum inhibitory concentrations (MICs) varied for each solvent; for instance, *E. coli* was inhibited by all extracts at 5 µg/ml, whereas at 1000 µg/ml, none of the extracts exhibited antimicrobial activity against *S. aureus*. Additionally, *S. mutans* and *C. albicans* were inhibited by the aqueous methanol extract at 1000 µg/ml, while the same concentration of petroleum ether and chloroform extracts did not affect the growth of *S. mutans*. Furthermore, concentrations ranging from 100 to 1000 µg/ml of all extracts inhibited *Klebsiella* species. Phytochemical tests indicated that the petroleum ether extract contains alkaloids and terpenoids, while the chloroform extract contains terpenoids only. In contrast, the aqueous methanol extract contained terpenoids, glycosides, phenolic compounds, flavonoids, tannins, and saponins. Gas chromatography-mass spectrometry (GC-MS) analysis identified various compounds in all extracts, highlighting similarities and differences in retention times (RTs) and peak areas.**

***Keywords*— *Antimicrobial Activity*, *Conocarpus* species, *Extracts*, *GC-MS*, *Solvents*.**

I. Introduction

Medicinal plants recognized as sources of drugs, traditional medicines, and antimicrobial agents [1]. The genus Conocarpus, belonging to the Combretaceae family, includes perennial and evergreen shrub species [2]. Two major species within this genus are: *C. erectus* and *C. lancifolius*. These plants are found in various regions around the world and hold significance in pharmaceutical biotechnology [3].Research has shown that extracted compounds from *Conocarpus* demonstrated activity against pathogenic bacteria [4]. Studies indicated that crude extracts from the leaves of *Conocarpus* species can inhibit the growth of certain pathogenic bacteria [5]. Moreover, these leaf extracts have exhibited effects against fungi and viruses, as well as antioxidant properties[6].Other researchers have reported that *Conocarpus* species are among the medicinal plants used to treat various diseases, including diabetes mellitus, syphilis, skin ulcers, anemia, and fever [7]. Bacterial resistance to antibiotics poses a serious public health issue. Plants represent a valuable resource for overcoming this challenge by providing new antibiotics to treat diseases caused by pathogenic microorganisms [8]. Antibiotic resistance has been identified as a threat to global health, with data indicating millions of deaths associated with this issue [9]. While hundreds of antimicrobial peptides have been discovered from various sources, including plants, many have not yet been developed into antibiotics. However, some antimicrobial agents have been utilized for treating diseases [10]. In light of this, the current study aims to test three crude extracts from the leaves of *Conocarpus* species produced using three different solvents. The objective is to evaluate their antimicrobial activity against certain microbial pathogens and to determine which solvent yields an extract with the greatest antimicrobial efficacy, along with an analysis of the phytochemicals present in the crude extracts.

II. Materials and Methods

A. Preparation and Extraction of Plant Sample

Healthy leaves were collected from *Conocarpus* species growing in the Thi-Qar Government, south of Iraq (Fig. 1). The leaves were thoroughly washed with tap water, followed by distilled water then left to dry at room temperature for three days. Once dried, the leaves were pulverized using a small mill to obtain a fine powder. Two hundred grams of this powder were placed in a suitable glass beaker (with a capacity of 1000 ml) and soaked in 700

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ml of absolute petroleum ether. The beaker was tightly covered with two layers of parafilm and left at room temperature for four days. Afterward, the filtrate was separated from the petroleum ether-soaked powder. To remove chlorophyll from the *Conocarpus* leaves, filter papers coated with 0.5 g of activated charcoal were employed as a thin layer. The petroleum ether evaporated from the filtrate until a solid substance was obtained. Simultaneously, the petroleum ether-soaked plant powder was processed under the same conditions to ensure complete evaporation and to eliminate any residue of the solvent, resulting in dried powder. For the next extraction step, 800 ml of absolute methanol was mixed with 200 ml of distilled water to create an 80% aqueous methanol solution. A volume of 700 ml of this solvent was added to a beaker containing the plant powder from which the petroleum ether had evaporated. This mixture was left for one week, following the same extraction method used for the petroleum ether. The dried powder obtained from the aqueous methanol extraction was then subjected to chloroform extraction, using the same steps as those for petroleum ether. Finally, the powder soaked in chloroform was discarded. All crude filtrate extracts produced from these solvents were stored in the refrigerator until needed.

Fig.(1): Tree of *Conocarpus* species.

B. Antimicrobial Activity

The crude extracts obtained from petroleum ether, aqueous methanol (80 %), and chloroform were separately dissolved in DMSO to prepare 10000 µg/ml solutions for each extract. These solutions were then sterilized using a Millipore (0.45 µm) syringe and tested against three bacteria: *S. aureus*, *S. mutans*, and *E. coli*, as well as the yeast *C. albicans*: tested identified microorganisms were obtained from Al-Imam Hussein Teaching Hospital in Thi-Qar Government, Iraq. The antimicrobial activity was assessed using the agar well diffusion method. Petri dishes containing nutrient agar were inoculated with 100 µL of a bacterial suspension (1.5×10^8 cells/ml) spread evenly on each dish. After the agar had dried, wells 7 mm in diameter were made in the center of each dish, and each well was filled with 100 µL of the 10000 µg/ml extract. *C. albicans* was tested at the same concentration and cell number but utilizing potato dextrose agar. The dishes were incubated at 37 °C for 2 days, after which the diameters of the inhibitory zones were measured in millimeters. This test was performed in triplicate.

C. Minimum Inhibition Concentrations (MICs)

This MIC test was conducted using the agar well diffusion method. Petri dishes filled with Mueller Hinton Agar (MHA) were used to test the extracts against *S. aureus*, *S. mutans*, *E. coli*, *Klebsiella* sp., and *C. albicans*. Each dish was inoculated with 100 µL of a 1.5 x 10^8 cells/mL suspension of each microorganism. Four wells, each 7 mm in diameter, were created in each Petri dish and loaded with four different concentrations of each extract: 1000, 100, 10, and 0.5 µg/mL. The dishes were incubated at 37 °C for 2 days, and the test was performed in triplicate.

D. Phytochemical Tests of Crude Extracts

The tests were conducted according to [11]. Each extract solution (ES) was prepared by dissolving it in the solvent used for extraction, and the tests were performed as follows:

E. Terpenoid Detection

1. **Salkowski's Test**: To conduct this test, mix 2-3 ml of the extract solution (ES) with 2 ml of absolute chloroform. Filter the mixture, and then carefully add a few drops of concentrated sulfuric acid along the side of the test tube without shaking it. If a reddish-brown color forms at the interface of the two layers, this indicates the presence of terpenoids. Conversely, if a golden yellow color appears, it suggests the presence of triterpenes.
2. **Copper Acetate Test**: For this test, treat 2-3 ml of the extract solution (ES) with a few drops of a 5% copper acetate solution. The appearance of a green color indicates the presence of diterpenes.

F. Alkaloids Detection

1. **Wagner's Test:** To perform this test, treat 2-3 ml of the extract solution with 2 ml of Wagner's reagent (prepared by dissolving iodine in potassium iodide). The presence of alkaloids is indicated by forming a brown or reddish-brown precipitate.
2. **Dragendroff's Test:** This test involves acidifying 2-3 ml of the extract solution with a single drop of sulfuric acid, followed by the addition of 0.5 ml of Dragendroff's reagent (a potassium bismuth iodide solution). The presence of alkaloids is confirmed if a red precipitate appears.

G. Glycosides Detection

**General test: ** Treat 5 ml of the ES with a few drops of 10% aqueous NaOH solution. If glycosides are present, a yellow color will be observed.

H. Phenolic Compounds Detection

1. **Alkali Test:** Add a few drops of 40% aqueous sodium hydroxide solution to 2 ml of the extract solution (ES). If flavonoids are present, a yellow-orange color will appear, which turns colorless upon adding a few drops of diluted acetic acid.
2. **Lead Acetate Test:** Add a few drops of 10% lead acetate solution to 2 ml of the extract solution (ES). If phenolic compounds are present, a white precipitate will form.

I. Tannins Detection

1. **Ferric Chloride Test**: To perform this test, add 1 ml of the extract solution (ES) to 2 ml of distilled water. Then, add 2-3 drops of a diluted 5% ferric chloride solution. A green to blue-green color indicates the presence of catechol

tannins, while a blue-black color suggests the presence of gallic tannins.
2. **Gelatin Test**: In this test, add a few drops of a 1% gelatin solution containing 1% sodium chloride to 2-3 ml of the extract solution. A white precipitate indicates the presence of tannins.

J. Saponin Glycosides Detection

1. **Foam Test:** Add 1 ml of the extract solution (ES) to a tube containing 2-3 ml of distilled water. Shake the mixture vigorously. If foam forms and persists for 10 minutes, saponins are present.
2. **Froth Test:** Dilute 1 ml of the extract solution (ES) with distilled water up to 20 ml. Shake the mixture in a graduated cylinder for 15 minutes. If a foam layer of at least 1 cm appears and persists for 15 minutes, saponins are present.

K. GC-MS Analysis

A small amount (1 mg) of each extract was dissolved in 5 ml of absolute methanol. The solution was then filtered using a Millipore filter syringe (0.45 µm). Next, 0.5 ml of the filtered solution was mixed with 4.5 ml of absolute methanol. Additionally, 0.5 ml of this new solution was mixed with another 4.5 ml of absolute methanol. The GC-MS analysis was conducted as described in reference (12), where 1 µL of each extract was analyzed using a MassHunter GC-MS device, following the protocol outlined in the accompanying table. The oven temperature began at 130 °C and was maintained for 6 minutes. It was then increased to 202 °C for 12 minutes before gradually rising to 230 °C over 2 minutes. The final temperature of 230 °C was held for 10 minutes.

Table 1. Parameters and conditions for GC-MS analysis used in detecting compounds in the extracts.

Parameters	Conditions
Injector	Equipped with split/spitless
Column of Restek Rtx-Wax	30 m × 0.32 mm i.d., 0.25 µm d.f.
Carrier gas	Helium
Flow rate	1 mL/min.
Injector initial temperature	230 ◦C
Oven temperature	130 ◦C increased to 230 ◦C
Operation of the interface	230 ◦C
Ionization energy	70 eV
Adjusting detector	Mass range: m/z 50–800.

L. Statistical Analysis

The statistical analysis was conducted using GraphPad Prism 5 software.

III. Results

A. Characteristics of Extracts and Their Antimicrobial Properties

The petroleum ether extract was noted to be a yellowish-green, sticky substance, while the aqueous methanol (80%) extract appeared as a brown powder. The chloroform extract was described as a greenish-yellow, sticky substance. A volume of 100 µL of each extract at 10000 µg/ml was prepared, and the aqueous methanol extract showed activity against *C. albicans*, *E. coli*, *S. aureus*, and *S. mutans*. The petroleum ether extract exhibited activity against all the mentioned microorganisms except for *S. mutans*. The chloroform extract only inhibited *S. aureus* and *E. coli* (Table 2 and Fig. 2).

Table 2. Antimicrobial activity of three crude extracts obtained from leaves of *Conocarpus* species against some microbial pathogens using 10000 µg/ml of each extract.

Type of extract	Inhibitory zones measured by millimeters (mm)			
	S. aureus	*S. mutans*	*E. coli*	*C. albicans*
A	18 ± 1	13 ± 1	21 ± 1	19 ± 1
P	13 ± 1	0	10 ± 1	15 ± 1
C	13 ± 1	0	23 ± 1	0

Values were significantly different at $P \leq 0.05$. The inhibition zone values are expressed as mean ± SD from three independent experiments. Note: The antimicrobial activity of petroleum ether and chloroform extracts against *S. mutans* is insignificant. A: 80% Aqueous methanol extract, P: Petroleum ether extract, and C: Chloroform extract.

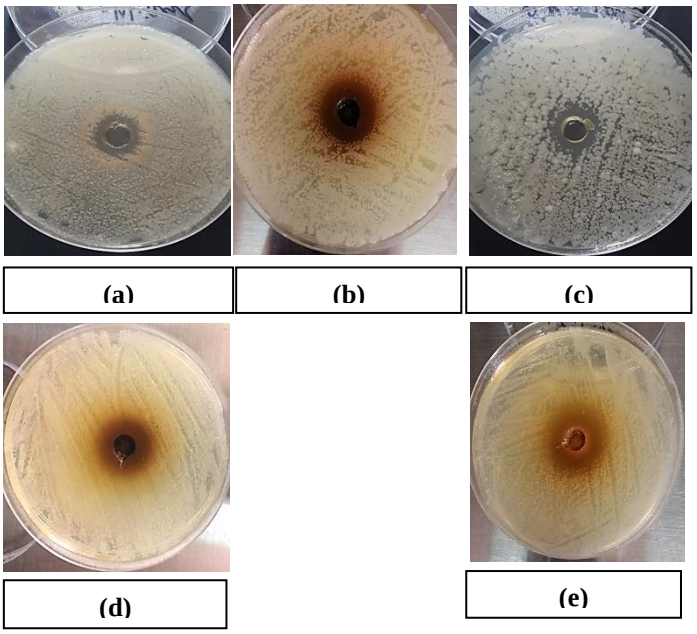

Fig.(2): Antimicrobial activity of two crude extracts obtained from the leaves of *Conocarpus* species against three microbial pathogens. (a) Aqueous methanol crude extract against *C. albicans*. (b) Aqueous methanol crude extract against *S. mutans*. (c) Petroleum ether crude extract against *C. albicans*. (d) Aqueous methanol crude extract against *E. coli*. (e) Aqueous methanol crude extract against *S. aureus*.

B. MIC Values

The MICs obtained from crude extracts of aqueous methanol, petroleum ether, and chloroform exhibited varying results. All extracts inhibited *E. coli* at a concentration of 5 µg/ml; however, a concentration of 1000 µg/ml did not show any antimicrobial activity against *S. aureus*. Furthermore, *C. albicans* and *S. mutans* were inhibited by the aqueous methanol extract at a concentration of 1000 µg/ml, while the same concentration of petroleum ether and chloroform extracts did not affect the growth of *S. mutans*. Additionally, concentrations ranging from 100 to 1000 µg/ml of all extracts were effective in inhibiting *Klebsiella* species (Table 3).

Table 3. MIC values of three crude extracts produced from leaves of *Conocarpus* species against some microbial pathogens using concentrations of each extract ranged from 5 to 1000 µg/ml.

Type of Extract	MICs (µg/ml)				
	S. aureus	*S. mutans*	*E. coli*	*Klebsiella* sp.	*C.albicans*
A	No effect	1000	5	1000	1000
P	No effect	No effect	5	100	No effect
C	No effect	No effect	5	1000	No effect

A: 80% Aqueous methanol extract, P: Petroleum ether extract, and C: Chloroform extract

C. Phytochemical Tests

The phytochemical tests indicated that the extract produced by aqueous methanol contained more compounds compared to those obtained from petroleum ether and chloroform. For instance, the methanolic extract was reported to include phenolic compounds and flavonoids (Table 4).

Table 4. Phytochemical tests reveal the presence of compounds in three extracts of *Conocarpus* species leaves.

Compounds	Extract produced by		
	P	A	C
Terpenoids	+V	+V	+V
Flavanoids	ED	+V	ED
Glycosides	ED	+V	ED
Phenolic compounds	ED	+V	ED
Saponin glycosides	ED	+V	ED
Tannins	ED	+V	ED
Alkaloids	+V	NT	NT

+V: Present. NT: No present, ED: The extract cannot be dissolved in the solution or solvent. A: 80% aqueous methanol extract, P: Petroleum ether extract, and C: Chloroform extract.

Table 5. The GC-MS analysis of the crude extract obtained from leaves of *Conocarpus* species using absolute petroleum ether.

Compounds	Peaks	R.Ts	Area	Quality
3-Tetradecene, (Z)-	1	6.785	3531596	96
2,4-Di-tert-butylphenol	3	9.218	548970	96
7-Hexadecene, (Z)-	4	10.333	5545156	99
1-Octadecene	6	12.732	3892688	99
Hexadecanoic acid, methyl ester	7	14.477	411443	98
Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	8	14.863	903481	99
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	9	15.174	228326	94
5-Eicosene, (E)-	10	15.45	1680855	99
1-Docosene	11	19.167	229784	91

Table 6. The GC-MS analysis of the crude extract obtained from leaves of *Conocarpus* species using 80% aqueous methanol.

Compounds	Peaks	R.Ts	Area	Quality
1-Tetradecene	1	6.76	2098413	97
2,4-Di-tert-butylphenol	3	9.209	527689	97
7-Hexadecene, (Z)-	4	10.325	4999404	99
1-Octadecene	6	12.732	3668814	98
Hexadecanoic acid, methyl ester	7	14.477	295430	95
Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	8	14.855	867157	99
9-Eicosene, (E)-	10	15.45	1585224	99
1-Hexacosene	11	19.166	272340	95

D. GC-MS Analysis

The GC-MS analysis revealed various retention times (R.T.s) and different areas for the tested extracts. This analysis detected some similar compounds across all extracts, while others varied. Examples of similar compounds include 2,4-di-tert-butylphenol, 1-octadecene, hexadecanoic acid methyl ester, and benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester. Additionally, 7-hexadecene (Z)- was found in petroleum ether and aqueous methanol extracts. Moreover, 5-eicosene (E)- was detected in the petroleum ether and chloroform extracts but was absent in the aqueous methanol extract. These findings are detailed in Tables 5, 6, and 7.

Table 7. The GC-MS analysis of the crude extract obtained from leaves of *Conocarpus* species using absolute chloroform.

Compounds	Peaks	R.Ts	Area	Quality
3-Tetradecene, (E)-	1	6.592	663215	96
2-Tetradecene, (E)-	2	6.76	747483	97
2,4-Di-tert-butylphenol	3	9.201	342277	96
Cetene	4	10.283	767040	97
2-Tetradecene, (E)-	5	10.333	3744688	98
1-Octadecene	7	12.732	3207383	96
Hexadecanoic acid, methyl ester	10	14.477	327945	98
Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	11	14.863	740992	99
5-Eicosene, (E)-	13	15.45	1350415	99
Octacosanol	14	19.175	227301	91

IV. Discussion

This study concluded that aqueous methanol (80%) is the most effective solvent for extracting compounds from the powdered leaves of the *Conocarpus* species, compared to petroleum ether and chloroform. The results align with the idea that selecting an appropriate solvent is crucial for successfully extracting compounds from plants [12]. This finding supports the aim of the present study, which was to identify a suitable solvent for extracting crude compounds from the leaves of *Conocarpus* species [13]. The solvents used in this study included two non-polar solvents (petroleum ether and chloroform) and one polar solvent

(methanol mixed with distilled water). The results indicated that the methanolic extract is the best option.

The findings of this research align with previous studies [13-14], which indicated powdered leaves of *Conocarpus lancifolius* Engl. extracted with methanol demonstrated significant antimicrobial activities. In the current study, extracts obtained using petroleum ether, aqueous methanol, and chloroform exhibited varying inhibition zones against the tested microbial species. The different susceptibility values observed in the tested species may be due to the presence of specific bioactive compounds in one extract compared to the others. It was noted that water and methanol are polar solvents capable of extracting polar compounds from the target substance.

Mixing water with a polar solvent increases the polarity of the solvent. Most bioactive compounds in plant matrices are medium-sized molecules, as these matrices contain aromatic π-electrons that are delocalized. As a result, these compounds tend to be highly polar. Scientists have observed that the choice of solvents for extracting dried plant powder depends on the polarity of the solute of interest. Methanol and water are polar solvents, while petroleum ether and chloroform are non-polar solvents [1]. Polar solvents, such as methanol and water, are highly effective in the extraction process. Specifically, methanol is known to extract more phenolic compounds from plants [1 -15]. This supports the observation that the aqueous methanol extract in the present study contained a rich content and demonstrated good antimicrobial activity compared to extracts produced with petroleum ether and chloroform. The increased polarity resulting from the mixture of water and methanol likely contributed to the extraction of polar compounds from the dried powdered leaves of *Conocarpus* species.

Relating to solvent polarity and bioactivity, the present study demonstrated that the extract obtained from petroleum ether yielded better results than that obtained from chloroform; however, both solvents are non-polar. It can be concluded that petroleum ether extracted more non-polar compounds from the powdered leaves of *Conocarpus* species because it is a more non-polar solvent than chloroform. Chloroform was used after petroleum ether and aqueous methanol, indicating that the extraction was performed sequentially using the same powdered leaves of the mentioned plant. The antimicrobial activity of an extract depends on the type of solvent used for extraction [16].Results from other researchers indicated that methanol produced extracts containing various phytochemicals, whereas chloroform extracted the least amount of these compounds [17]. In the current study, the extract produced by chloroform exhibited the least antimicrobial activity and phytochemical content, which aligns with the findings of other researchers. Therefore, polarity plays a significant role in the content of an extract; for instance, 80% aqueous methanol yielded a richer collection of phytochemicals compared to petroleum ether, which resulted in the lowest content [18].

Polar compounds can be extracted using polar solvents, while non-polar compounds are obtained from non-polar solvents. This necessitates careful selection of solvents for the extraction process [19-21]. This explains why the aqueous methanol crude extract in the current study demonstrated stronger antimicrobial properties and higher phytochemical content compared to the extracts produced using petroleum ether and chloroform. Studies have confirmed that the Concocarpus plant possesses antimicrobial activities against both bacteria and fungi. One example of a compound found in this plant is tannin, which was detected in its crude extract [22]. This finding aligns with the phytochemicals identified in the present study. Additionally, flavonoids, phenols, and alkaloids were detected in C. erectus, and these compounds showed activity against multi-drug-resistant *Staphylococcus aureus* [23- 26].

The crude extracts from the current study demonstrated the presence of various phytochemicals and exhibited antimicrobial activity against the tested microbial pathogens. Related studies found that compounds derived from the leaves of *C. lancifolius* and *C. erectus*, such as tannins and saponins, showed antimicrobial properties [27-28], which supports the findings of the present research. Additionally, a local study [29] highlighted the antimicrobial effects of leaf extracts from *C. erectus*, which grows in the Thi-Qar government, located in southern Iraq—the same area where the tree used in this current study was sourced. However, it was noted that the chloroform extract of *Conocarpus* species leaves did not affect the growth of certain pathogenic filamentous fungi [30]. Regarding solvent polarity, it was observed that water and methanol are polar solvents that extract polar compounds within the target substance. Adding water to a polar solvent increases its polarity [15]. In contrast, petroleum ether and chloroform are non-polar solvents that can extract non-polar compounds [13-15].

V. Conclusions

The 80% aqueous methanol solution is the most effective solvent for extracting antimicrobial compounds from powdered leaves of *Conocarpus* species when compared to petroleum ether and chloroform. However, further studies are necessary to identify and characterize the bioactive compounds present in the crude extracts.

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CONFLICT OF INTEREST

Author declares that he has no conflict of interest.

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