ISSN: 03875547
Volume 45, Issue 01, February, 2022

Antibacterial activity of alkaloid compound Methoxy phenyl –Oxime ($C_8H_9N0_2$) isolated and purified from leaf of *Conocarpus lancifolius* Engl.

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Keywords:
methoxyphenyl-Oxime,
conocarpus lancifolius,
Antibacterial activity,
Alkaloid compounds

ABSTRACT

Identification of the useful components present in medicinal plants can lead to a better understanding of their pharmacokinetics, toxicity, metapolism, and simple isolation resulting in development of the best and most effective medications. *Conocarpus lancifolius* plant was collected on March, 2020 from Basrah governorate, Southern of Iraq. Biochemical test for alkaloid compounds was done for all parts of plant alone (leaf, fruit, root, flower) that appeared the existence of alkaloid in leaves only. Extraction for alkaloid compounds was done by using three solvents (methanol, water, dichloromethane) that revealed methanol is superior to other two solvents with a significant differences at level of p>0.05. of a value reached (1.6%g) whereas, values for both water and dichloromethane solvents were reached (0.96%) (0.146%) respectively. GC-MS-analysis was carried out for leaf methanolic alkaloid extract that appeared one main peak of alkaloid compound, methoxy phenyl oxime $C_8H_9N0_2$ at R.T reached 5.2min with area% reached 42.02%. Identification, separation and purification for MPO compound was done by using High Performance Liquid Chromatography (HPLC) technique that resulting in appearance of one peak in 280 wave length at R.T reached 10.4 min. Antibacterial activity of purified MPO compound that using three concentrations(100,150,200 µl) revealed that (200 µl) cons was superior of a value reached(20.9mm) to each other two cons (150,100 µl) that reached (18.6mm), (17.6)respectively with a significant differences at a level of p>0.05.thus, Antibacterial activity (as average) against *B.subtillus* that reached 21.55 mm was superior to that against each of *E.coli* and *K. pneumonia* that reached 19.44mm and 17.11mm respectively with a significant differences at a level of p>0.05,whereas ,there is no significant differences of antibacterial activity against *S. aureus*.ith values of MIC that reached (35,55,95,95, µl) for *B.subtillus, S.aureus E.coli* and *K. pneumonia* respectively. Hemolytic cytotoxicity of the MPO molecule, on the other hand, was found to be 1.22 percent in healthy human erythrocytes.

1. INTRODUCTION

There are two species of Conocarpus in the Combretaceae family, and they're native to tropical regions of the planet. Both Conocarpus erectus and Conocarpus lancifolius are common mangrove species, although only Conocarpus erectus may be found in a large stretch of the southern Red Sea coast. Phytoremediation in arid climates could benefit from this fast-growing and drought-resistant tree species [20]. As secondary metabolites, the active chemicals present in many medications can be found in plants such as alkaloids, [1]. Additionally, different sections of the plant provide extracts of C. erectus with bioactivity against Gram positive and Gram negative bacteria (Seeds, Stem, Leave and bark) [13]. C. lancifolius extract also has antibacterial activity that demonstrated high diameter of inhibitory zone by disc diffusion method against different bacteria species at concentrations of 10 to 200 g/mL of extracted alkaloid [3]. Because of its biological action against a variety of harmful microorganisms, Conocarpus [15]. Antiprotozoal efficacy is also demonstrated by Conocarpus ethanolic extract. Several polyamines discovered in C. lancifolius were associated with the type, severity, and length of environmental stress that the plant had been exposed to. Our understanding of how this plant responds to environmental stressors like drought and salinity will aid in its management and long-term viability [4]. In some places outside of its native range, the Conocarpus tree is utilized as a medicinal plant for the treatment of flu, conjugative inflammatory conditions, diarrhea and fever. For example, *C. Lancifolius* can be used for vomiting, bleeding, orchitis, skin ulcers and syphilis [17], [18]. It is widely accepted that bacterial infections are an important public health issue. In addition, multidrug resistance has led to an increase in mortality and morbidity. New antibacterial medications are therefore required, and plants are considered to be a major source of innovative natural chemicals as a result [16]. Because of its propensity to thrive in harsh environments, it has spread over southern and central Iraq over the last decade. This study aimed to identification and separation of alkaloid compounds existence in *C.lancifolius* and evaluation its antimicrobial activity.

2. Material and method

2.1 Plants collection

Conocarpus lancifolius was collected on March, 2020 from Basrah governorate, Southern of Iraq. Identification of the field collected plant was authenticated as C. lancifolius by plant Taxonomist Prof Dr.Sahar abd-alabas, ,Collage of Science, Department of Biology , University of Basrah.

Classification of Conocarpus lancifolius
Kingdom :Plantae
Phylum :Tracheophyta
Class : Magnoliopsida
Order :Myrtales
Family :Combretaceae
Genus : Conocarpus (L.)
Species: Conocarpus lancifolius Engl.

Figure-1 Conocarpus lancifolius Engl..

2.2 Preparation of plant to extraction

After plant was classified, using tab water, the entire plant was properly sanitized. any contaminates and then shade dried after separated all parts of plant alone and the dried parts ground to fine powder through mechanical grinder and then stored in tight plastic bags labeled for study.

2.3 Preliminary Biochemical test for alkaloid compounds

Two Biochemical tests were done for Methanolic extract (Hot continuous extraction) of each part alone (Leaf,root,fruit,flower) for studied plant, *Conocarpus lancifolius* for detection of alkaloids by using two tests:
1--Dragendorff s reagents: 1ml of reagent was added drop by drop to the extract, formation of reddish-brown or orange precipitate indicates presence of alkaloids [14].
2-Mayer reagent: 1 ml of reagent is added to the extract drop by a creamy precipitate implies that alkaloids are present [12].

2.4 Solvent Extraction

Hot continuous extraction by Soxhelet using two inorganic solvents (Methanol and water) and one organic solvent (Dichloromethane) for *C.lancifolius* (leaves) to choose the best solvent for alkaloids extraction.

2.5 Extraction of total Alkaloids

According to [14]. A soxhlet apparatus 250 ml volume was used to extract 20 g of plant dry powder for 24 h in a continuous extraction with 80 percent of methanol. The filtrate was filtered and then the filtrate was concentrated under vacuum at 45C until the solution reached to 10 ml and washed with 10 ml chloroform

three times, the pH value of the extract reached (pH=2) To acquire the entire alkaloid extract, the chloroform component was dried, the dried extract was weighed, and it was stored at 4 degrees Celsius for future research.

2.6 Screening for alkaloid compounds by using Gas chromatography-mass spectrum (GC-Mass) analysis

Screening of plant extracts were done in Basra oil company –Nihranbinomar- Laboratory. For Methanolic alkaloid extract of *Conocarpus lancifolius* (leaves) that appeared existing of alkaloid compounds thus, for detecting their alkaloid types and structure by using modified method [23] by GC-.MS analysis. Spectrometer Agilent HP- 5MS gas chromatograph connected to an Agilent 5977A mass spectrometer with a mass detector (5% Phenyl methyl siloxen), 30m ×0.25mm × 0.25 mm ID of capillary column. The temperature of injector was 40 0C maintained for 5 min then raised gradually to 300 0C at rate of increment 10\min. Helium gas 99.99% used as mobile phase at flow rate of 1ml\min. an injection volume of 1 uL. The fragments ranged in size from 45 to 450 Da, and the scan interval was 4 minutes. The mass spectra were collected at 70 ev. The solvent delay was four minutes, and the GC-MS run time was 45 minutes. A split injection was used to administer the samples (50:1) It was determined that the mass spectral scan range was 45 to 650 (m/z).

2.7 Identification, Separation & Purification of alkaloid compounds by using High Performance Liquid Chromatography (HPLC) technique

HPLC analysis was carried out using modified method [21] LC-W100A HPLC (USA) system connected to LC-UV100 plus UV detector with manual injectors. Data interfered using PC with (les (x86) \HPLC SYSTEM) Separation was performed through Exformma technologies Column Arcus EPC18 5um, 4.6 x 250mm, isocratic mobile phase used for analysis of leaf methanolic alkaloid extract for *C.lancifolius* consist of (acetonitrile: water50:50), with 1ml/min flow rate at 25 C, and pressure of 100 p.s.i , injection volume was 10 ul. 0.05 mg\ml sample of leaf methanolic alkaloid extract that was filtered by (125mm) filter paper before injection, run time was for 10 min for each run, and detection was conducted at (280) wave length. Thus, Modified method used to separate and purify methoxy phenyl oxime compound by HPLC [7]. The eluted mobile phase during the appearance of the identified peak of methoxy phenyl oxime with recorded retention time in compare to study of [8] that identified methoxy phenyl oxime by HPLC at R.T reached 10 min at the same conditions. Isolated mobile phase portions then undergo HPLC analysis to confirm purity of isolated.

2.8 Antibacterial activity of purified MPO compound

2.8.1 Bacterial Source and Identification

The microbiology lab at Abingasoan-Hospital collected and identified all of the bacteria employed in this investigation as human pathogens, as stated in the table (3).

Table (3): Source of isolates of pathogenic bacteria has been investigated.

Pathogenic bacteria	Source
Escherichia coli	Stool
Klepsiella pneumonia	Blood-
Staphylococcus aureus	Ear soap
Bacillus subtilis	Blood

2.8.2 Kirby-Bauer disc diffusion method

The Kirby-Bauer disc diffusion susceptibility test [19] method was used to measure the sensitivity of the bacterial strains against alkaloid compound (methoxy phenyl oxime). On the surface of solid media plates, 105 CFU/mL of the tested bacteria were distributed in a 1 mL solution. Plates were inoculated with 20 L of the MPO compound put into filter paper discs with a diameter of 5 mm. For 24 hours, the plates were kept at 37°C. Millimeters were used to measure the widths of the inhibition zones (IZs). The antibacterial activity of

the MPO compound was tested against the tested bacterial strains at concentrations of 200, 150, and 100g/mL. There was a positive and negative control; the discs were saturated with the 20 mL DMSO solution for the negative control. Triplicate experiments were carried out.

2.8.3 Minimum inhibitory concentrations (MIC)

Methoxy phenol oxime, a chemical isolated from the leaves of Conocarpus lancifolius, was diluted in sterile Mueller Hinton Broth (MHB) in 9 6-well microplates [11], and the MICs were measured by filling all wells with 50 L sterile MHB with slight modifications. Both the sterility and growth controls contained Oxoid MHB (Sigma-Aldrich) in the sterility well, while both the MHB and the test organism was present in the growth well for the latter purpose. The micro-plate was covered and incubated overnight at 37°C with 100 percent relative humidity after the bacterial suspension (105 CFU/mL) was added to each row (except for the sterility control). Each well was treated with 50 L of a 0.2 mg/mL solution of piodonitrotetrazolium violet (Sigma-Aldrich) to see if growth was being inhibited by a decrease in color reaction intensity.

2.8.4 In vitro Determination of Cytotoxicity

In the lab Hemolysis was used to test the cytotoxicity of alkaloid substance on human erythrocytes (RBCs) in vitro (methoxy phenyl oxime). As indicated in (Bouma.,2002), blood from healthy and nonsmokers was used in this study, with the following modifications: An alkaloid substance with 50 micrograms per milliliter (mg/ml) concentration was typically introduced to 0.2 milliliters of blood and mixed thoroughly for 5 seconds before 20 milliliters of water were added to prevent excessive hemolysis. The mixture then was centrifuged for 10 minutes at 3000 RPM. Finally, 540 nm optical density (O.D.) measurements were made. Aside from that, this test is designed to assess how much hemolysis is caused by MPO compound 100 percent hemolysis. We were able to accomplish total hemolysis (100%) by diluting the mixture, centrifuge it, and then measure the O.D. at 540 nm. Following the completion of the absorption measurement, the following equation was used to compute the percentage of hemolysis: At 100% H–AS, hemolysis percent is calculated as follows: (AT–AS) Test solution's ability to adsorb AS: Normal saline absorption A100% H: Absorption of hemolysis at 100%.

3. Results and discussion

3.1 Preliminary Biochemical test for alkaloid compounds

As the results shown in table 1- the plant C. lancifolius appeared existing of alkaloid compounds in leaves only by giving positive test for two Mayer &Dragendorff s reagents that corresponded with study.

3.2 Solvent extraction

The results for percentage of alkaloid yield extract in C. lancifolius as shown in table- 2 appeared that methanol is superior to other two solvents with a significant differences at level of $p>0.05$. of a value reached (1.6%g) whereas, values for both water and dichloromethane solvents were reached (0.96%) (0.146%) respectively. methanol extract of *C.lancifolius* contain a bioactive compounds twice from that of aqueous extract [2]. The stronger extraction capacity of methanol have been produced many active constituents responsible for several biological activities. Amount of a bioactive compound affected by many factors of extraction conditions like temperature, time of extraction, besides type and concentration of a used solvent.

Table --1: Biochemical testes for alkaloid compounds detection.

Plant	Part of plant	Alkaloid test

		Dragendorff reagent	Mayer reagent
Conocarpus lancifolius	Fruit	-	-
	Flower	-	-
	Leaf	+	+
	Root	-	-

Table 2-: Percentage of alkaloid yield extracts in *C.lancifolius* by using three solvents (methanol, water, dichloromethane).

	% of Alkaloid yield extracts g/100mg		
Plant	Methanolic Extraction	Water Extraction	Dichloromethane Extraction
C.lancifolius (leaf)	1.600	0.96	0.146

RLCD =0.369

3.3 GC-MS analysis for leaf Methanolic alkaloid extract of Conocarpus lancifolius.

As shown in table 3-3 the results of GC-MS analysis for leaf methanolic crude alkaloids extract of *Conocarpus lancifolius* revealed the presence of one type of alkaloid compound that is Methoxy phenyl oxime at R.T reached 5.2 min with area percentage reached 42.02%, and molecular weight 551.0g/mol as appeared in table-3 where, this is the first study that confirmed the presence of a novel methoxy phenyl oxime compound in this plant that found to be existing in few plants by GC-MS analysis such as methanolic leaves extract for both *Urtica dioica* and *Alstonia scholaris* also the study of [8] revealed the existence of MPO compound in seeds of *Momordica charantica*.

Table -3-: GC-MS analysis for leaf Methanolic alkaloid extract of Conocarpus lancifolius.

Name of alkaloid compounds	Retention. Time (min)	Formula	M Wt. g/mol	Area percentage %
methoxy phenyl oxime	5.2	C8H9N02	551.1	42.02%

ISSN: 03875547
Volume 45, Issue 01, February, 2022

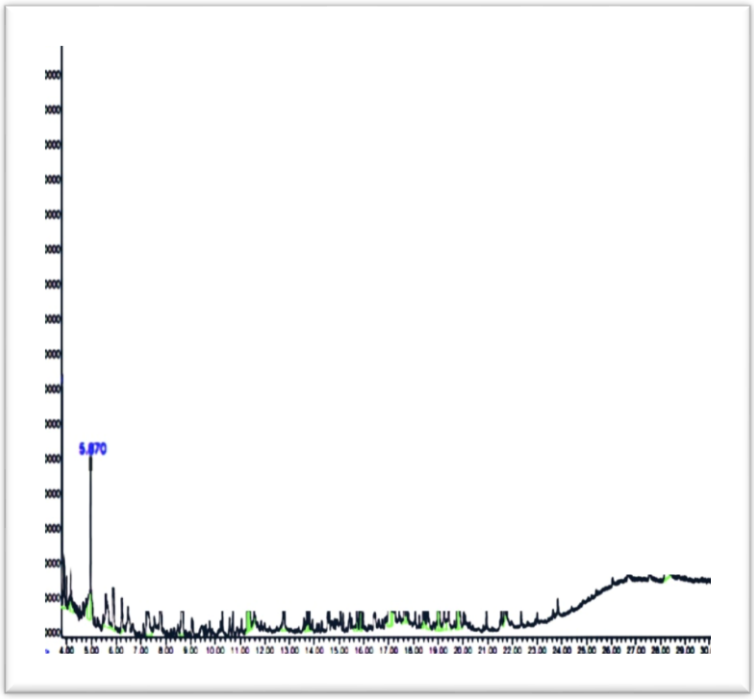

Figure 2: GC-MS chromatogram for leaf methanolic alkaloid compounds in *C.lancifolius*.

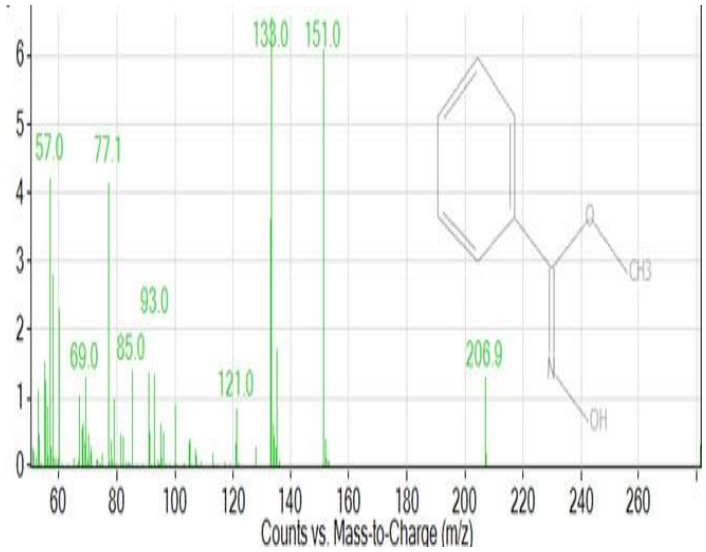

Figure 3- Mass-spectrum of alkaloid compound Methoxy Phenyl Oxime C8H9N02 that existing in leaf methanolic extract.

3.4 Isolation, Purification and Identification of alkaloid compounds by HPLC technique

HPLC analysis of methanolic leaf alkaloid extract of *C lancifolius* resulted in separation of one major peak at R.T reached 10.2min at wave length of 280nm as shown in figure-1. that identifying as methoxy phenyl oxime by comparing with R.T for methoxy phenyl oxime compound that separate in a study of [8] at a R.T reached (10.4min).

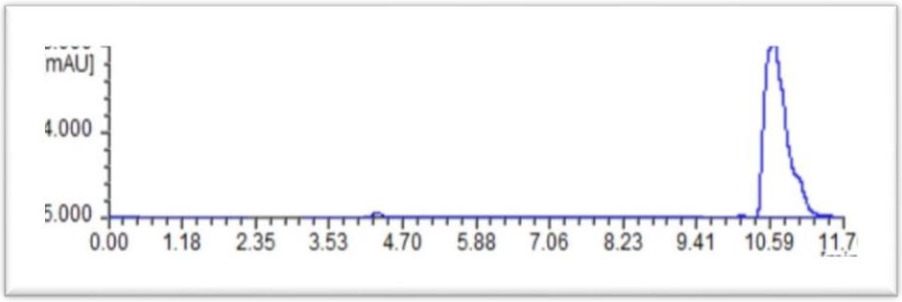

Figure HPLC chromatogram for leaf methanolic alkaloid extract of C. lancifolius

3.5 Antibacterial activity of MPO compound

Based on results from three trials (200,150,100), the antibacterial activity of MPO substance against both gram positive and gram negative bacteria was found to be as indicated in table 4. This includes staphylococci aureus, Bacillus subtillus, and Escherichia coli (200 µl) cons was superior of a value reached (20.9mm) to each other two cons (150,100 µl) that reached (18.6mm), (17.6) respectively with significant differences at a level of $p<0.05$.thus, antibacterial activity (as average) against *B.subtillus that reached 21.55 mm was superior* to that against each of *E.coli* and *K. pneumonia that reached 19.44mm and 17.11mm* respectively with a significant differences at a level of $p<0.05$,whereas ,there is no significant differences of antibacterial activity *against S. aureus*. This result corresponded with the study of [6] that appeared antibacterial activity of MPO compound against *S. aureus*, *S. epidermis and E. coli* that confirmed methoxyphenyl-oxime and other oximes behave as antibiotics so, methoxy phenyl oxime has a high diameter of inhibition zone against studied bacteria in compare with that for leaf crude extract of *C. lancifolius* that has a little diameter (12 mm<) of inhibition zone as in study of [22]. Also minimum inhibition concentration (MIC) was carried out for MPO compound against four studied bacteria as shown in table-5 that appeared lowest value of MIC against *B.subtillus* that reached 35mg, and then against *S. aureus* of a value reached 55mg whereas ,MIC value against both, *E. coli* and *K. pneumonia* were 95mg. According to the results shown in table -6- of Hemolysis cytotoxicity for purified alkaloid compound methoxy phenyl oxime on RBCs showed that there is no Hemolysis cytotoxicity because MPO has low value of erythrocyte disruption effect that percentage value of RBCs hemolysis in current study for methoxy phenyl- Oxime was1.22%. Previous study of showed no Hemolysis cytotoxicity on RBCs of leaf aqueous extract of *C.lancifolius*.

Table 4- Antibacterial activity of purified alkaloid compound methoxy phenyl oxim

Inhibition zone /mm					
Alkaloid compound	**Con. /** µg	***S.aureus***	***B.subtillus***	***E.coli***	***K.pneumonia***
Methoxy phenyl oxime	200	22.33	22.66	20.66	18.33
	150	21.00	21.33	19.33	18.00
	100	18.00	20.33	18.33	18.00
Positive	30	21.49	20	0	19.77

control (Gentamycin)					
Negative control DMSO.	20	0	0	0	0

RLSD=MPO (Bacteria)=1.34, (Con)=1.64.

Table-5: Minimum inhibition concentration (MIC) for MPO compound against four studied bacteria

		MIC% µg/ml			
Plant	**alkaloid compounds**	***S. aureus***	***E. coli***	***K. pneumonia***	***B. subtillus***
Conocarpus lancifolius	Methoxy phenyl-Oxime	55	95	95	35

Table -6 Absorbance of human erythrocytes after treating with, methoxy phenyl oxime in *C. procera* at 450nm.

Treatment type	**Absorbance at 450nm**
Methoxy phenyl -Oxime (50 µg/ml)	0.027
Normal saline	0.008
% Hemolysis	1.562

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