ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 8, Issue 1, 2015

ISSN - 0974-2441

Research Article

IN VITRO ANTIBACTERIAL ACTIVITY AND PHYTOCHEMICAL ANALYSIS OF *ORMOCARPUM COCHINCHINENSE* EXTRACTS ON NOSOCOMIAL INFECTION CAUSING BACTERIA

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Received: 26 September 2014, Revised and Accepted: 29 October 2014

ABSTRACT

Objective: The present study was aimed at evaluating the antibacterial activity and phytochemical analysis of the leaf extracts of *Ormocarpum cochinchinense*.

Methods: The leaf powder was extracted by sequential extraction method using chloroform, acetone, and methanol. Preliminary phytochemical analysis of the extracts was done using standard methods. The dried extracts were screened for antibacterial activity using disk diffusion method and the minimum inhibitory concentration (MIC) method.

Results: The acetone extract showed the presence of flavonoids, tannins, steroids, phlobatannins, and saponins. Methanol extract contained flavonoids, tannins, phlobatannins, saponins, and terpenoids. The chloroform extract contained steroids. The chloroform extract did not show any activity, whereas the acetone extract showed significant activity against all the bacteria with a MIC value of 0.080 mg/ml against *Escherichia coli* (ATCC 25922) and *Klebsiella pneumoniae* (ATCC 15380) and 0.18 mg/ml against *Staphylococcus aureus* (meticillin resistant). The methanol extract showed very less activity.

Conclusion: The present study has proved the antibacterial potency of *O. cochinchinense* for the first time. This plant is a potential source of antimicrobial agents of the future.

Keywords: Antibacterial activity, Phytochemical analysis, Minimum inhibitory concentration, *Ormocarpum cochinchinense*, Acetone extract.

INTRODUCTION

Nature has been the source of medicinal agents for thousands of years and a large population of the people living in the developing countries almost exclusively uses traditional medicines. Over the past decades, antibacterial drugs have been used and misused extensively leading to the development of multi-drug resistant bacteria. This has resulted in global health security emergency that is rapidly outpacing available treatment options [1]. In India, this has become a great public health concern because of the widespread use and availability of practically all antimicrobials over the counter and the absence of definite guidelines for pathogens of public health importance [2].

According to "antimicrobial resistance, global report on surveillance" by WHO in 2014, bacteria commonly causing infections in hospitals because of antibiotic resistance are: (a) *Escherichia coli* that causes urinary tract infections and blood stream infections, which shows resistance to third-generation cephalosporins, including resistance conferred by extended spectrum beta-lactamases (ESBLs), and to fluoroquinolones. (b) *Klebsiella pneumonia*, responsible for pneumonia, blood stream infections, and urinary tract infection, which shows resistance to third-generation cephalosporins, including resistance conferred by ESBLs, and to carbapenems. (c) *Staphylococcus aureus* -causes wound infections and blood stream infections, which is resistance to beta-lactam antibacterial drugs (methicillin, methicillin-resistant *S. aureus* [MRSA]).

This multiple resistance exhibited by bacteria has led to the search for novel antibiotics or antibacterial compounds. Since plants are the natural reservoirs of bioactive secondary metabolites, our attention has turned to screening medicinal plants for their potential activity. It is significant that in India, most of the traditionally used medicinal plants have not yet been validated scientifically. One such plant is *Ormocarpum cochinchinense* (Tamil: Elumbotti and Kattumuringai) that belongs to the *Fabaceae* family. Traditionally, the leaves are eaten fresh or prepared into a medicated candy (lehiyam) and then consumed. It helps to cure chest pain. The leaves are used for setting bone fractures and for nervous pain. The roots are used in the treatment of lumbago and paralysis. Its wound healing property alone has been studied [3] and it is significant that there is very little scientific evidence for its medicinal and antimicrobial properties. Hence, an attempt has been made to evaluate its antibacterial property against three pathogenic bacteria causing hospital infections as mentioned by WHO in its Global Report on Surveillance in 2014 [1].

METHODS

Bacterial cultures

Two standard strains of each of the three bacteria mentioned by WHO [1] which commonly cause infections in hospitals, namely *E. coli* (ATCC 25922 and ESBL 3904), *K. pneumonia* (ATCC 15380 and ATCC 109), and *S. aureus* (ATCC 25923 and MRSA), were selected.

Plant collection

The selected plant *O. cochinchinense* was collected from Chengalpattu after four or five showers. The plant was identified by taxonomist Dr. G. Jeya Jothi and a voucher specimen (LCH 308) was deposited in the Department of Plant Biology and Biotechnology, Loyola College, Chennai. The leaves were washed, air dried until brittle, ground into a fine powder, and then stored in airtight containers for further analysis.

Extraction

Plant extraction was done by sequential extraction method using chloroform, acetone, and methanol. The powder to solvent ratio was taken as 1:4. The extraction was carried out for 72 hrs for each solvent after which the extract was filtered using a Whatman filter paper and allowed to dry. The dried solvent-free extract was collected and stored in small amber bottles at 5°°C until further analysis.

Phytochemical analysis

Qualitative phytochemical analysis was done for all the extracts. Standard procedures described by Sofowora [4], Trease and Evans [5], and Harborne [6] were followed for alkaloids, carbohydrates, proteins, saponins, flavonoids, terpenoids, cardiac glycosides, anthraquinones, tannins, and phlobatannins.

Test for alkaloids

An amount of 50 mg of extract was dissolved in 5 ml of dilute hydrochloric acid and filtered, and the filtrate was used for analysis.

Mayer's test: To 2 ml of filtrate, two drops of Meyer's reagent was added. Appearance of white or creamy precipitate confirmed the test.

Wagner's test: To 2 ml of filtrate, few drops Wagner's reagent was added. A reddish-brown precipitate indicated the test as a positive.

Test for carbohydrates

A few milligram of extract was dissolved in 5 ml of water, filtered, and used for analysis.

Molisch's test: To 2 ml of filtrate, two drops of α-naphthol were added and shaken well, and 1 ml of concentrated sulfuric acid was added along the sides. A violet or red-brown ring appears on standing indicated the presence of carbohydrates.

Fehling's test: 2 ml of filtrate is boiled on the water bath to which 1 ml of Fehling's A and Fehling's B solutions were added. Appearance of red precipitate indicated the presence of sugars.

Barfoed's test: To 1 ml of filtrate, 1 ml of Barfoed's reagent was added and heated on the water-bath for 2 minutes. A red precipitate confirmed the test.

Test for proteins and amino acids

A few milligram of filtrate was dissolved in 5 ml of distilled water, filtered, and used for analysis.

Biuret test: To 2 ml of filtrate, 0.5 ml of copper sulfate was added. Violet/pink color indicated the presence of proteins.

Xanthoproteic test: To 2 ml of filtrate, 0.5 ml of concentrated nitric acid was added. Formation of yellow color confirmed the test.

Ninhydrin test: Two drops of ninhydrin solution is added to 1 ml of filtrate. Appearance of violet or purple color indicated the presence of amino acids.

Test for saponins

Foam test: 5 mg of extract was dissolved in 3 ml water and shaken well for 5 minutes. Persistent foam formation indicated the presence of saponins.

Test for flavonoids

NaOH test: To 1 ml of the extract dissolved in water, 2 ml of 10% NaOH was added. The solution appears yellow color, which then disappears on adding few drops of dilute hydrochloric acid confirmed the test.

Ammonia test: To 1 ml of the aqueous filtrate of the extract, 0.5 ml of dilute ammonia was added and shaken well. Yellow coloration of the ammonia layer indicates the presence of flavonoids.

Test for terpenoids and sterols

Salkowski test: To 2 ml of extract in chloroform, few drops of concentrated sulfuric acid were added. Development of reddish brown interface indicates the presence of terpenoids and the development of green fluorescence indicates the presence of sterols.

Test for cardiac glycosides

Keller–Kiliani test: To few milligram of the extract, 1 ml of glacial acetic acid was added, and few drops of 5% ferric chloride were added. When 1 ml of concentration sulfuric acid is added, brown ring appears in the interface, which indicates the presence of cardiac glycosides.

Test for anthraquinones

Borntrager's test: Few amount of the extract was dissolved in dilute sulfuric acid and filtered. To the filtrate, benzene was added and shaken well. The organic layer is separated to which ammonia is added slowly. The ammoniacal layer shows pink to red color due to the presence of anthraquinone.

Test for tannins

Ferric chloride test: To 2 ml of the extract dissolved in water, 1% ferric chloride was added in drops. Appearance of dark green or deep blue color indicated the presence of phenols.

Test for phlobatannins

To 1 ml of extract dissolved in water, 1 ml of 1% HCl was added and boiled. Deposition of red precipitate indicates the presence of phlobatannins.

Antimicrobial assay

Preparation of bacterial innoculum

The test organisms were inoculated in Mueller-Hinton broth and incubated overnight. The concentration of the culture was adjusted to 10^4 cfu/ml- using 0.5% McFarland as standard. This concentrated culture was used for disk diffusion method and for determining minimum inhibitory concentrations (MIC) using microtiter technique.

Disc diffusion assay

Antibacterial activity was done using disc diffusion method [7]. Extracts were diluted in di methyl sulfoxide (DMSO). 25 µl of the dilutions was loaded onto sterile discs and dried. The discs loaded with plant extract were placed on the petriplates inoculated with the test organism. The plates were incubated for 24 hrs and the antimicrobial zones formed were measured, and the results were tabulated. The concentration of the extracts tested was 2.5 mg, and the plates were done as triplicates, and the mean value was taken as the zone of inhibition, which was expressed in millimeters (mm). Streptomycin (10 µg), vancomycin (10 µg), and methicillin (10 µg) were taken as a positive control, and DMSO was taken as a negative control.

Determination of MIC

MIC was done for the extracts that showed antibacterial activity. Serial microtiter plate dilution method developed by Eloff [8] was followed with little modification. 100 mg of the extract was dissolved in 1 ml of DMSO from which 100 µl was loaded on the first well and serially diluted with sterile water. 25 µl of test organism was added to each well. The final well was loaded with 50 µl of test organism. Streptomycin and Vancomycin were used as a positive control.

The microtiter plates were incubated at 37 °C for 24 h. After incubation, 40 µl of 0.2 mg/ml P-Iodonitrotetrazolium (INT) violet was added to all the wells and incubated for half an hour to one hour to note the color change. INT is converted to formazan in case of bacterial growth, giving rise to red color. The lowest concentration in which red color is not seen is taken as the MIC of the extract. The MIC values were expressed as mg/ml.

RESULTS

Phytochemical analysis

The crude extracts of chloroform, acetone, and methanol were screened for phytochemicals, and the results were given in Table 1. The chloroform extract showed the presence of sterols alone. Carbohydrates and terpenoids were found only in methanol extract, whereas anthraquinones was only present in acetone extract. Alkaloids,

flavonoids, cardiac glycosides, tannins, and phlobatannins were present in both acetone and methanol extracts.

Antibacterial activity

The chloroform, acetone, and methanol extracts were tested for their antibacterial activity by disc diffusion method. The chloroform extract did not show any antibacterial activity against any of the bacteria. The results are shown in comparison to the antibiotics that were used (Fig. 1).

The acetone extract showed significant activity against all the organisms except for *Klebsiella pneumoniae* (ATCC 109), which was also resistant to vancomysin and methicillin but slightly susceptible to streptomycin, which showed a zone of 12 mm.

The acetone extract showed an average zone of 13 mm, which was higher than methicillin (average zone of 7.75 mm) and was on par with vancomycin (average zone of 13.5 mm) and streptomycin (average zone of 13.3 mm). The methanol extract showed minimum activity showing zones ranging from 7 mm to 8 mm. The chloroform extract did not show any activity.

MIC

MIC was performed for acetone and methanol extracts. Streptomycin and vancomycin were taken as standards. Appearance of pink color was observed after ½ hr of adding INT. Those wells that still had live bacterial cells turned red and those wells in which bacterial cells have been killed appeared colorless. The results were tabulated in Table 2.

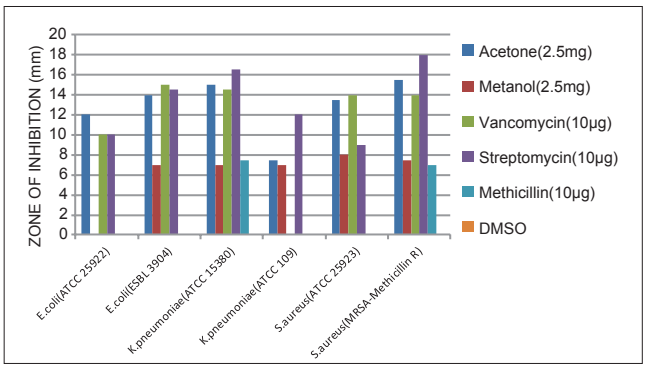

Fig. 1: Zone of inhibition against test organisms

Table 1: Phytochemical analysis of extracts

S. No.	Phyto-constituent	Chloroform	Acetone	Methanol
1	Alkaloids	–	+	+
2	Carbohydrates	–	–	+
3	Proteins and amino acids	–	–	–
4	Saponins	–	+	+
5	Flavonoids	–	+	+
6	Terpenoids	–	–	+
7	Sterols	+	–	–
8	Cardiac glycosides	–	+	+
9	Anthraquinones	–	+	–
10	Tannins	–	+	+
11	Phlobatannins	–	+	+

The acetone extract showed MIC value of 0.080 mg/ml against *E. coli* (ATCC 25922) and *K. pneumoniae* (ATCC 15380) cultures, which was as potent as the standard controls, streptomycin and vancomycin. Against *S. aureus* (ATCC 25923) and *S. aureus* (MRSA), it showed a MIC of 0.18 mg/ml, which was also a significant value when compared to the antibiotic standards.

K. pneumoniae (ATCC 109) was the only organism against which both the extracts and the vancomycin standard could not be effective in smaller concentrations. All except streptomicin that showed a MIC of 0.080 mg/ml were ineffective at lower concentration against *K. pneumoniae* (ATCC 109) strain. The methanol extract showed MIC values of 6.2 mg/ml, 12.5 mg/ml, 6.2 mg/ml, 25 mg/ml, 12.5 mg/ml, and 12.5 mg/ml, respectively, against the six pathogens.

DISCUSSION

According to WHOs Global Report on Surveillance on antibiotic resistance over the last 30 years, no major new types of antibiotics have been developed [1]. Moreover, bacteria are rapidly becoming resistant to all the major antibiotics throughout the world [1]. This led to a crisis situation, which has turned our attention to the traditional medicines.

In the present study, a diverse range of phytochemicals, such as alkaloids, flavonoids, tannins, and phlobatannins, are present in acetone as well as methanol extracts, which can account to the bioactivity of the crude extracts [9]. Terpenoids found in the methanol extract are the most abundant antimicrobials of plant origin [10]. The acetone extract showed the best activity against all bacterial cultures which is in acceptance with earlier studies that shows that acetone was the best solvent for screening and isolating antimicrobial compounds [11].

The acetone extract showed significant activity against all the bacteria except *K. pneumoniae* (ATCC 109), which showed high resistance. MIC values against *E. coli* (ATCC 25922) and *K. pneumoniae* (ATCC 15380) were 0.080 mg/ml, which were equivalent to that of the positive controls namely streptomycin and vancomycin. Moreover, a MIC value lower than 0.1 mg/ml is considered as significant internationally [12].

Earlier attempts by various researchers to study the antibacterial activity of *Ormocarpum* genus plants present in Africa have proved to be successful. *Ormocarpum trichocarpum* leaf extract has demonstrated a broad spectrum antibacterial activity [13]. Moreover, Ormocarpin and other flavonoids have been isolated from the root bark of *Ormocarpum kirkii*, which has potential antibacterial and antimalarial activity [14,15].

Hence, the present study has demonstrated the antibacterial potential along with the presence of various phytochemicals in the crude extracts of *O. cochinchinense*. The antimicrobial activity of the extract is in par with the antibiotics that have been used in this study. Now, there is a need for investigating the bioactive compound or compounds from the plant, which can become a source of future antimicrobials [16].

Table 2: MIC values of crude extracts and antibiotics

S. No.	Bacteria	MIC (mg/ml)			
		Acetone	Methanol	Streptomycin	Vancomycin
1	*E. coli* (ATCC 25922)	0.080	6.2	6.2	0.080
2	*E. coli* (ESBL 3904)	0.3	12.5	0.080	0.080
3	*K. pneumoniae* (ATCC 15380)	0.080	6.2	0.080	0.080
4	*K. pneumoniae* (ATCC 109)	3.12	25	0.080	6.2
5	*S. aureus* (ATCC 25923)	0.18	12.5	0.080	0.080
6	*S. aureus* (MRSA)	0.18	12.5	0.080	0.080

MIC: Minimum inhibitory concentration, ESBL: extended spectrum beta-lactamases, *E. coli: Escherichia coli, K. pneumonia: Klebsiella pneumonia, S. aureus: Staphylococcus aureus*, MRSA: Methicillin-resistant S. aureus

CONCLUSION

The present study has proved the antibacterial potency of *O. cochinchinense* for the first time against those bacteria that have become a major threat in hospital infections. Hence, it is a potential source of antimicrobial agents of the future. Further studies should focus on separation, isolation, and validation of the bioactive compounds and to test their antibacterial potency.

ACKNOWLEDGMENTS

The authors are grateful to Rev. Dr. G. Joseph Anthony Samy, Principal, Loyola College, and Professor L. Antoine Lebel, Head of the Department of Plant Biology and Biotechnology, Loyola College, for their constant help and continuous encouragement.

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