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RESEARCH ARTICLE

IN VITRO ANTIBACTERIAL ACTIVITY OF COPPER POD/YELLOW FLAME TREE OR PEELAGULMOHAR *(PELTOPHORUMROXBURGHII)*

***,[1]Tanveer Abbas, [1]Adil Anwar Bhatti, [1]Asma Saeed, [2]Farhana Tasleem, [2]Iqbal Azhar and [3]Zafar Alam Mehmood**

[1]Department of Microbiology, University of Karachi 75270, Karachi Pakistan
[2]Department of Pharmacognosy, Faculty of Pharmacy, University of Karachi
[3]Colorcon Limited, Cross ways, Victory way, Kent, Dart ford, England

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ABSTRACT

Drugs are the basic requirement to ensure the better quality of life thus various natural products from different sources has played an essential role. Plants including their different parts and exudates have been also used as natural antimicrobials as well as natural medicines to treat various medical problems, more than this, plants are the cheapest source of antimicrobials. With the passage of time microorganisms are getting resistance to various previously effective antibiotics. Pure, ethanol, and hexane extracts of *Peltophorum roxburghii* (copperpod) has shown significant activity against *Staphylococcus aureus, Enterococcus feacalis, Pseudomonas aeruginosa*and *Escherichia coli* in agar well diffusion method as well as micro titer broth dilution method, which contributes to use plant extracts as initial material to design drugs effective to the organisms used in the study.

INTRODUCTION

Today the drugs are the most important requirement in the field of successful treatment of various diseases including microbial origin and other etiology, thus to fulfill this need people are using various natural products from different resources including i. Terrestrialherbs and Land plants, ii. Different secretions of marine lives, iii. Fermentation yields of microorganisms and iv. Animal based Products. These all means provide a series of active components that then lead to the development of numerous drugs. Among all the above sources, plants are the most accessible to obtain antimicrobials (Norman *et al*., 1985; Hong-Fang *et al*., 2009). All these natural yields act against microorganisms, preventing their pathogenesis. The discovery of antimicrobial began with the observation that one microorganism prevent another (Baumberg *et al*., 1995). Plants are being used by men from the beginning of their existence, and many nations in the previous history are known for the use of plants as treatment of diseases (Marjorie Murphy Cowan, 1999 and http://www.faculty. ucr.edu/~ legneref/botany/medicine.htm).

Corresponding author: Tanveer Abbas,
Department of Microbiology, University of Karachi 75270, Karachi Pakistan.

The discovery and application of antimicrobials can be classified in to three periods as: pre antimicrobials era, antimicrobial era, and today! Pre antimicrobial era is characterized by high mortality in life threatening diseases, on contrary antimicrobial era shows comparatively lesser mortality while today the antimicrobials are the most moneymaking class of pharmaceutical companies! (Powers, 2004). A large population of the world relies on different plants to use them as treatment purpose thus they are really called "backbone" of traditional medicine, as they are unique source of drugs (Ahvaz *et al*., 2012; Kaushik *et al*., 2010).

Different parts of plants have been used to derive medicinal and antimicrobial components thus it gives great opportunity to introduce new drugs (Petrovska, 2012; http://www.infoplease.com/dk/science/encyclopedia/medicinal-plants.html). Although now a day's many people mostly prefer modern medicine but even those pills or capsules and other formulations are also driven from plants thus traditional medicine and allopathic goes on parallel (http://www.monographs.iarc.fr/ENG/Monographs/vol82/mono 82-6A.pdf; Bassam Abdul Rasool Hassan, 2012). Medicinal plants now have become an active class of drugs discovery due to deficiency of drugs in pharmaceutical industry, newly

emerging pathogens and resistance of microbes to previously actively working drugs (Mahady, 2005). Thus medicinal plants have great potential to treat any medical problem (http://www.liveandfeel.com/medicinal-plants). Extracts of some plants are very effective in their pure form while others need to process to make them as safe as to be non toxic to men (Olayiwola Akerele, 1991). High cost and access of allopathic medicines lead people to turn towards traditional cure of diseases more over allopathic medicines have side effects while medicinal plants provide relatively safest components (http://prr.hec.gov.pk/Chapters/302S-1.pdf).

Importance of medicinal plants can be categorized in various fields including economically inexpensive (http://agriinfo.in/default.aspx?page=topic&superid=2&topicid=1408), easily available because most of the medicinal plants grow wildly thus effortlessly accessible, progress in pharmaceutical industry as they are important source of drugs although synthetic drugs are fast acting but have more side effects on contrary medicinal plants are less toxic even act slowly comparatively (http://agriinfo.in/default.aspx?page=topic&superid=2&topicid=1408; http://www.dawn.com), therapeutic use due to containing a number of contents effectively working even different parts of the same plant have effect against completely different area of the body systems on the other hand many different plants can be used for a single infection (http://www.ehow.com/about_6743739_history_-scope-importance-medicinal-plants.html), mass cultivation i.e. cultivation offers the opportunity to preserve not only economically significant wild plants in their natural habitats, but also of providing farmers with new crops and significance in research areaand also use in cosmetics (http://www.agr.gc.ca/eng/science-and-innovation/science-publications-and-resources/resources/canadian-medicinal-crops/introduction-to-medicinal-plants/?id=1300832855227). *Peltophorum roxburghii* is one of the plant specie originated in Indo-Malayan region and found from Sri Lanka to Australia. (Zoë Gardner *et al*., 2004) It is suitable to use as a fodder even tree is used as fuelwood and in India, it is a source of pollen for the dammer bee (*Trigona iridipennis*). *P. pterocarpum* is widelyappreciated for its shade providing feature, due to its dense spreading crown (http://greengearsupm.blogspot.com/2014/01/peltophorum-pterocarpum.html). It belongs to family *Fabaceae* with synonym *Peltophorum pterocarpum* (http://www.ville-ge.ch/musinfo/bd/cjb/africa/details.php? langue=an&id=127869). Preferred common name of the plant is copperpod (www.cabi.org/isc/datasheet/39510). It possesses various medicinal properties including antimicrobial activity (Muhammad Mohtasheem ul Hasan *et al*., 2012; Syed Muzammil Ahmed *et al*., 2012). The present study of the selected plant extracts and its pure compound (bergenin) have been carried out to observe its antibacterial activity *in vitro* against different species, and to compare the effect of pure compound, hexane extract, and ethanolic extract.

MATERIALS AND METHODS

Test organisms

In the study clinical isolates *Staphylococcus aureus, Enterococcus sp., Pseudomonas aeruginosa, Salmonella typhi,Klebsiella pneumonia, Acenatobacter sp., Proteus sp.,*and *Escherichia coli*were used. All Organisms were sub cultured and maintained on nutrient agar (Oxoid) plates and slants at 37^{o}c. Gram staining was done to check the purity of cultures.

Microbiological Media

Microbiological Media including Nutrient agar, MHA, and Nutrient broth (Oxoid) were used.

Chemicals

DMSO (Dimethyl sulfoxide), McFarland index 0.5, and Normal Saline (for bacterial culture suspension).

Plant extracts preparation

Leaves of *Peltophorum roxburghii*were soaked first in hexane for fifteen days. Filterit and the filtrate was evaporated under reduced pressure by means of rotary evaporator,the plant material was resoaked in ethanol and the same procedure was repeatedwith ethanol (Farhana Tasleem *et al*., 2015).

Extract Dilution

In order to obtain particular concentration 0.3g of ethanol, pure and hexane extracts were dissolved in 3ml of DMSO by vortex thus 0.1g/ml, finally achieving 10%. The extract was first filtered using membrane filter (Corning) 0.45µ then saved in a McCartney and kept in refrigerator at 4°C until used.

Standard antimicrobial

Mother tincture of *Berberis vulgaris*

Antimicrobial activity analyses

Conventional antimicrobial activity analyses such as agar well diffusion test (Holder and Boyce, 1994) and broth dilution method (Paul *et al*., 2014) are considerably demanding processes for new antimicrobial agent discovery. Antibacterial activity of different types of extracts and a pure compound were determined against Gram positive and Gram negative isolates was assessed by these methods.

Agar Well Diffusion Assay

PRINCIPLE: The antimicrobials present in the plant extract are allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. The resulting zones of inhibition will be uniformly circular as there will be a confluent lawn of growth. The diameter of zone of inhibition can be measured in millimeters (http://shodhganga.inflibnet.ac.in/bitstream/10603/1458/11/11_chapter3.pdf). Petri plates containing 25-30ml Muller Hinton medium were seeded with 24hr culture of bacterial strains after solidification. Suspensions of the test organisms were made in normal saline and 10^{8}cells/ml were maintained by using McFarland index 0.5. Wells were prepared by using alcohol dipped sterile borer of 7mm in diameter by maintaining suitable space between each well. 100 µL (10% v/v or w/v) of plant extracts were added with the help of micropipette in

different wells in the plate and left it at room temperature for about 30 min. The plates were then incubated at 37°C for 24 hours. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well and expressed in mm as its antimicrobial activity. Each test was run in triplicate and repeat (n=6).

Determination of MICs by Microtiter broth dilution/ Micro Dilution method

PRINCIPLE: Dilution susceptibility testing methods are used to determine the minimal concentration of antimicrobial needed to inhibit or kill the microorganism. This can be achieved by dilution of antimicrobial in either agar or broth media. Antimicrobials are tested in log2 serial dilutions (two fold) (http://shodhganga.inflibnet.ac.in/bitstream/ 10603/ 1458/ 11/11_chapter3.pdf).The selected bacterial strains were inoculated onto nutrient agar slants to grow overnight. Overnight cultures were suspended in sterile normal saline and 10^8cells/ml was maintained by using McFarland index 0.5. This is the bacterial sample to be evaluated for antimicrobial sensitivity or resistance. 100µl of Muller Hinton broth was dispensed into all wells of a microtiter plate. The plate was labeled, as each row for a particular bacterial culture. 100µl of antimicrobial was pipetted into the wells in column 1 (far left of plate). Using the multi pipettor set at 100µl, the antimicrobial was mixed into the wells in column 1 by sucking up and down at least 3-4 times. Do not splash. 100µl was withdrawn from column 1 and added to column 2. This makes column 2 a twofold dilution of column 1. After mixing up and down 3-4 times, 100µl was transferred to column 3. The procedure was repeated down to column 11 only.

The same set of tips can be used for the entire dilution series. 100µl was discarded from column 11 rather than putting it in column 12. With the smaller multipipettor set to 10µl, bacterial culture suspension was dispensed into all wells except well 11, thus well 11 contained medium and plant extract only thus acting as sterility control and blank for the plate reader, containing only medium and antimicrobial. Bacterial culture suspension was added to column 12 i.e. column 12 contains medium and bacterial culture only for comparison.

This protocol was followed for all the selected antimicrobials in separate microtiter plates. All the plates were incubated at 37^{o}C for 24 hours. The plates were scanned with an ELISA reader (Magellan software). MIC of each extract was taken as the lowest concentration or highest dilution that did not give any visible bacterial growth or the lowest concentration of extract that reduces, by more than 50% or 90% for MIC_{50} or MIC_{90}respectively. At this concentration or dilution extracts were bacteriostatic.

Time kill Assay

Grow culture of *E. coli* (over night) in nutrient broth. Next dayculture was matched with Macfarlane index to give rise to 10^8 cells per ml. 10ml sterile nutrient broth was taken in test tube and only 10µl was discarded then 10µl culture was added that diluted it to 10^{-3} or 1: 1000. At last 1ml diluted culture and 0.1ml extract was transferred into a well dried and cleancuvettes finally readings for absorbance were takenat 600 nm using spectrophotometerat 0minutes, 30 minutes, 60 minutes, 120 minutes, 240 minutesand 1440 minutes. Similar procedure was followed for all the extracts.

RESULTS

The results of antimicrobial susceptibility testing in terms of zone of inhibition are shown in Table 1. Theabove data reveals that only *Escherichia coli*and*Pseudomonas aeruginosa*are susceptible to all of the three different preparations of the plant extracts, while *Acenatobacter sp., Proteus sp.,Klebsiella pneumonia,* and *Salmonella typhi* are showing resistance to the selected plant, and *Staphylococcus aureus* and *Enterococcus feacalis*shows susceptibility to pure compound (bergenin) and ethanol extract of the plant, but resistance to hexane extract. Above results for well diffusion method were further confirmed by micro titer broth dilution method to check the lowest concentration of the extracts to be effective to inhibit the bacterial growth. The results of MIC are given in Table 2.
The above results showed that pure compound of the plant has worked effectively against *E. coli, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa* as they were inhibited at very less concentration of 0.78, 0.195, and 1.56 mg/ml respectively, while other organisms are responding to the compound at same concentration 6.25 and 3.125 mg/ml.

Table 1. Zones of inhibition

	Zone of Inhibition (avg) using Agar Well Diffusion Method (mm)			
	pure compound	ethanol extract	hexane extract	*Berberis vulgaris*
Organisms				
Staphylococcus aureus	10.5	10	ND	21
Enterococcus feacalis	11	12	ND	16
Pseudomonas aeruginosa	15	12	14	19
Klebsiella pneumonia	ND	ND	ND	11
Salmonella typhi.	ND	ND	ND	15
Escherichia coli	11	12	10	15
Acenatobacter sp.	ND	ND	ND	10
Proteus sp.	ND	ND	ND	11

Table 1 shows average of triplicate and repeat inhibition zones of eight clinical isolates against Bergenin, Ethanol, and Hexane extracts of *Peltophorum roxburghii* in comparison to *Berberis vulgaris* obtained in Agar Well Diffusion Method. ND: Not Determined

Table 2. Minimum Inhibitory Concentrations

Organisms	MIC(mg/ml)			
	pure compound	ethanol extract	hexane extract	*Berberis vulgaris*
E. coli	0.78	25	6.25	3.125
E. feacalis	6.25	25	3.125	6.25
Klebsiellapneumoniae	0.195	25	3.125	1.56
Salmonella typhi	6.25	25	1.56	6.25
Pseudomonas aeruginosa	1.56	25	1.56	6.25
Acenatobacter sp.	3.125	12.5	3.125	3.125
S. aureus	6.25	12.5	1.56	25
Proteus sp.	3.125	25	6.25	12.5

Table 2 shows minimum inhibitory concentration of Bergenin, Ethanol, and Hexane extracts of *Peltophorum roxburghii* in comparison to *Berberis vulgaris* against eight clinical isolates determined by Micro titer broth dilution method.

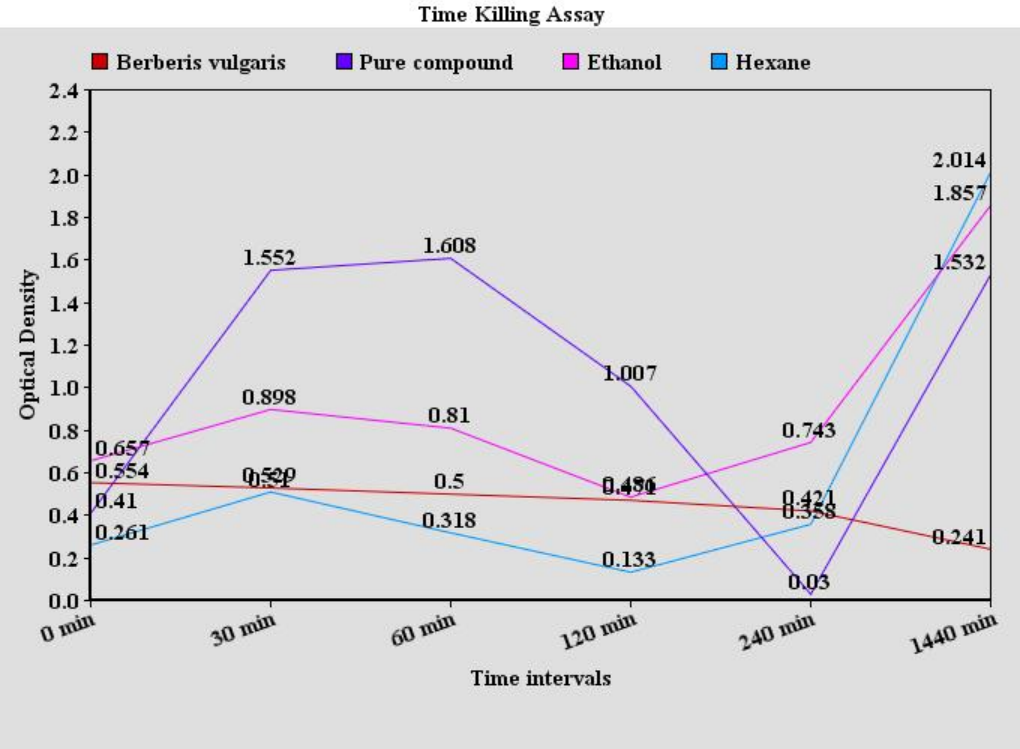

Figure 1 shows Time-Kill Evaluation of the Bergenin, Ethanol, and Hexane extracts of *Peltophorum roxburghii* in comparison to *Berberis vulgaris* against *E. coli*

Figure 1. Time killing assay

In case of hexane extract *Salmonella typhi, Pseudomonas aeruginosa,* and *S. aureus* were inhibited by the same concentration of 1.56 mg/ml and ethanol extract is working at very high concentration (25mg/ml) more or less against all of the experimental organisms. T. TEST p values, comparing each extract with standard show *P (> 0.05, <<0.05, and >0.05).* The above figure shows that pure compound of the plant began to workafter 60 minutes and after 240 minutes its efficacy is completely eliminated, ethanol extract showing better results than pure compound (bergenin) as it appears to be effective after 30 minutes but at 120 minutes its efficacy also abolished finally hexane extract showing its activity from 30 minutes but at 120 minutes it gradually lost its activity and stop to work effectively.

DISCUSSION

Emergence of resistance is widely increasing through out the microbial world which results in weakening the work of drugs and antimicrobials. Among all of the selected bacterial species most of them including *Staphylococcus aureus, Klebsiella sp., Acenatobacter sp.,* and *Enterococcus faecalis*have shown resistance to various antibiotics thus they are becoming threat to precious lives. This problem requires a great concern in the development of new antimicrobials to treat infections, and plants have played an important role to be used as potent antimicrobials. *Peltophorum roxburghii*has shown its activity against threatening bacterial strains, as *Staphylococcus aureus*was appeared to be sensitive by hexane extract even at very less concentration (1.56 mg/ml) but no zone of inhibition

was observed in well diffusion method which might be due to insolubility as some antimicrobials are not very soluble so they do not dissolve well in agar plate that makes them unable to diffuse very far a way from well and Z.O.I might look misleadingly small even the compound is very effective. Thus to get more detailed results MIC by broth dilution method is necessarily performed. Similar results were also obtained in some of the other tested bacterial species during the present study that no activity was shown in the first assay while good results in second one which might be due also to largeinoculum size during first assay. MICs of bergenin and hexane extracts range from 0.78 to 6.25 mg/ml especially against *Pseudomonas aeruginosa* both extract preparations were highly active as displaying comparatively larger zones of inhibition 15 and 14 mm respectively more over effectively working at very low concentration (1.56 mg/ml) in micro dilution method and generally it is assumed that the bigger the zone of inhibition the more effective the test antimicrobial. Ethanol extract is effective at 25 mg/ml against most of the organisms which is much higher than that of the other two types of extracts. In the present study killing assay against *E.coli* was also performed to ensure about the efficacy of extracts that at what time they start to kill it and at which exact time effectiveness of the extracts decreases and then completely ends. In this regard similar curves were obtained in ethanol and hexane extracts while pure compound (bergenin) although effective at very less concentration i.e. 0.78 mg/ml but started to work at/after 60 minutes and within approximately two hours it stop to work and the remaining cells of *E.coli* started to grow as it might be possible that some of the cells remain which survive further. But over all results show that *Peltophorum roxburghii* may serve as a starting material for drug design to treat infections caused by the tested bacterial species. All the results were compared with natural antimicrobial rather than that of any synthetic antibiotic. MIC of pure compound is exactly similar to the *B. vugaris*for *Enterococcus fecalis* and *S. typhi*and also in time killing assay absorbance in ethanol and hexane extracts at two to three positions is coinciding with that of the standard in figure1.

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