Biomedical Research 2017; 28 (1): 134-139 *ISSN 0970-938X*
www.biomedres.info

Evaluation of antimicrobial activity of ethanolic extracts of *Azadirachta indica* and *Psidium guajava* against clinically important bacteria at varying pH and temperature.

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Abstract

The present study was carried out to evaluate the antimicrobial activity of ethanolic extracts of *Azadirachta indica* and *Psidium guajava* against four clinically important bacteria namely *Staphylococcus aureus*, *Salmonella typhi*, *E. coli* and *Pseudomonas aeruginosa*. The antimicrobial activity of extracts was done with agar well diffusion assay in plates containing MHA media. A fresh bacterial culture of 100 µl containing approximately 1 × 10^6 CFU/ml of test microorganism was inoculated onto media plates and spread homogeneously. Wells of 6 mm in diameter were punched off in MHA plates with pH 6.0, 7.5, and 9.0, filled with different quantities of extracts in various combinations, and incubated for 24 h at 35°C and 37°C. The antimicrobial activity of ethanolic extracts showed that all the combinations of extracts were effective against the test microorganisms. The pH 6.0 and temperature 35°C with concentration of each extract at 25 mg/µl was found to be the inhibitoriest against all the bacteria tested. The diameter of zones of inhibition at pH 6.0 exhibited by *S. aureus* were in the range of 17-30 mm, *E. coli* (18-26 mm) and *S. typhi* (9-12 mm) and the zone of inhibition for *P. aeruginosa* was observed between 19-23 mm. The antimicrobial activity of the ethanolic extract was reduced when the same combination of extract concentration was tested at 37°C. The antimicrobial activities of both the extracts were severely affected negatively at pH 9.0. The results contained in this study indicate that these plant extracts have enormous antimicrobial potential, and may be exploited for the treatment of various infectious diseases.

Keywords: *Psidium guajava, Azadirachta indica,* Plant extracts, Ethnomedicine, Infectious diseases, Antimicrobial activity.

Accepted on May 04, 2016

Introduction

Antibiotics provide the basis for the fungal and bacterial infections therapy. The discovery of antibiotics and making use of them as chemotherapeutic agents has made the medical fraternity to believe that they will eradicate various infectious diseases. However, indiscriminate use of antibiotics in human and veterinary healthcare systems has lead to the emergence of multi-drug resistant (MDR) strains of different groups of microorganisms [1,2]. The emergence and dissemination of MDR bacteria has made chemically synthesized antibiotics ineffective for the treatment of infectious diseases caused by

such bacteria [3]. These circumstances have propelled the researchers and scientists to explore new antimicrobial substances from various sources such as medicinal plants [4]. There are many studies that have described different type of plants such as herbs, shrubs and trees with the aim of knowing their phytoconstituents and using them for the treatment of various diseases as possible alternatives to the synthetic drugs [5]. The screening of plants for medicinal purposes represents a serious effort to discover newer, safer, and possibly more effective drugs with the potential of fighting pathogenic bacteria and fungi [6]. The green medicines are widely believed as safe and dependable in contrast with expensive synthetic drugs that have undesirable side effects along with beneficial effects [7]. The plants have been in use in traditional medicine worldwide since long time but are still understudied, particularly in clinical microbiology [8]. In past few decades, the curiosity to evaluate plants possessing antimicrobial, antifungal, anti-inflammatory activity for various diseases has grown many folds and a large number of biologically active compounds have been characterized [9]. Several studies have established that many plants contain substances like peptides, tannins, alkaloids, essential oils, phenols, and flavonoids among others, which have antimicrobial properties [10,11].

Azadirachta indica is an evergreen tree commonly found in Indian subcontinent, which is famous by the local name Neem. Neem tree contains a large number of biologically active compounds that are chemically diverse and structurally complex. More than 140 compounds including alkaloids, flavonoids, triterpenoids, phenolic compounds, carotenoids, steroids, ketones azadirachtin and nimbin have been isolated from different parts of neem [12,13]. Almost every part of this tree is used in different diseases as traditional drug. The tree and its extracts have also been reported to possess insecticidal, anti-viral, anti-fungal and anti-bacterial properties [14]. Extracts of Neem seed have been found to inhibit bacterial pathogens involved in eyes and ear infections [15]. *Psidium guajava* is member of the family Myrataceae, which is very common in the tropical countries and known by the English name Guava. The Guava is a phytotherapic plant, which contains active components that help to treat various diseases like malaria, gastroenteritis, vomiting, diarrhea, dysentery, wounds, ulcers, toothache, coughs, sore throat, inflamed gums, and a number of other conditions [16,17]. The components present in Guava include lectins, phenols, tannins, flavanoids, essential oils, fatty acids, vitamins, etc. However, most of the medicinal properties of the Guava are attributed the presence of flavanoids. The aim of this study was to evaluate the antimicrobial activity of ethanol extracts of *Azadirachta indica* and *Psidium guajava* to establish if they are effective in inhibiting the growth of bacteria such as *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, and *Pseudomonas aeruginosa* at different pH and temperature.

Materials and Methods

Sample collection and extraction

Fresh leaves of *P. guajava* and *A. indica* were collected from the vicinity of University area. Leaves were washed gently under tap water and left to dry at room temperature for 2 days, the leaves were then crushed separately to make powder. 50 gram of the powder of *P. guajava* and *A. indica* were mixed separately with 200 ml ethanol in conical flasks. The flasks containing extracts were heated on water bath for 1 h and placed at room temperature for 5 days. The flasks were manually shaken daily to obtain maximum extraction. After 5 days, each extract added to falcon tubes and centrifuged at 4000 rpm for 10 min to separate the supernatant. The supernatant containing extracts of *P. guajava* and *A. indica* were transferred into pre-weighed beakers and were left to dry completely on water bath at 60°C to obtain an ethanol free extract residue of *P. guajava* and *A. indica*.

Preparation of stock solutions

The dry powdered extracts of *P. guajava* and *A. indica* were dissolved separately in dimethyl sulfoxide (DMSO) to get the final concentration of 0.5 mg/µl for each extract.

Test organisms

Microbial strains were obtained from the microbiology laboratory of PCSIR, Lahore, (Pakistan). In total, four microorganism were tested against the above mentioned plant extracts in which one was Gram positive *Staphylococcus aureus* and three were the Gram negative namely *Escherichia coli*, *Salmonella typhi* and *Pseudomonas aeruginosa*. The strains were maintained on nutrient agar slants at 4°C.

Preparation of culture media

Mueller Hinton Agar (MHA) media (Oxoid, UK) was prepared by suspending 38 g in 1000 ml of distilled water. The media was sterilized by autoclaving at 121°C for 15 min and poured into sterile Petri plates at around 50°C. To observe the effect of pH on the growth of tested bacteria, pH was adjusted by adding 0.1M HCL or 0.1M NaOH into the media. The pH was adjusted at 6.0, 7.5, and 9.0 with the help of digital pH meter.

Antimicrobial assay

The antimicrobial activity of ethanol extracts of *P. guajava* and *A. indica* was done using the agar well diffusion assay as described earlier [18]. Petri plates of 90 mm diameter were poured with MHA media and allowed to solidify to make a base layer. The MHA plates were marked to divide into required parts and labelled for specific organism, extract name, and pH. A fresh bacterial culture of 100 µl containing approximately 1×10^6 CFU/ml. CFU/ml of test microorganism was inoculated onto MHA media plates and spread homogeneously using a glass spreader; the plates were incubated for 15 min at 37°C to complete dryness of media

surface. Wells of 6 mm in diameter were punched off with the help of sterile borer in MHA plates. Wells were then filled with the plant extract solution (ranging 10 µl–25 µl) in the combinations. Petri plates were placed for 30 min in refrigerator for diffusion of extracts and then incubated at temperatures 35, 36 and 37°C for 24 hours. At the end of the incubation period, the zone of inhibition (including well diameter) was measured. The experiment was carried out thrice independently in duplicate and the mean of all the readings is mentioned in the results. Gentamycin 5 µg/disc was used as positive control and DMSO (solvent) used as negative control.

Results

The ethanolic extracts of *P. guajava* and *A. indica* were tested for their antimicrobial activity in combinations of different concentrations ranging from 17.5 mg/µl-25 mg/µl and at varying pH and temperature. The tested plants have been in use as folk medicine and were familiar to the local people of Punjab, Pakistan. The results of the antimicrobial activity of ethanolic extracts showed that all the concentrations were effective against tested microorganisms with varying zones of inhibition. The pH 6.0 and temperature 35°C with concentration of each extract at 25 mg/µl was found to be the inhibitoriest against all the bacteria tested. At pH 6.0, the diameter of zones of inhibition exhibited by *S. aureus* were in the range of 17-30 mm, *E. coli* (18-26 mm) and *S. typhi* (9-12 mm) and the zone of inhibition for *P. aeruginosa* was observed between 19-23 mm. At pH 6.0 and temperature 35°C, the combined extract of *P. guajava* and *A. indica* when tested at concentration 25 mg/µl each, was found most inhibitory to *S. aureus* and *E. coli* showing zone of inhibition of 30 mm and 26 mm respectively (Table 1). This combination further inhibited growth of *P. aeruginosa* fairly well (20 mm zone diameter); however, it was less effective in inhibiting the growth of *S. typhi* where zone diameter was observed only 11 mm. When pH and extracts concentrations were kept constant (pH 6.0 and 25 mg/µl each extract) and temperature raised by 2°C (37°C), the antimicrobial activities against the test isolates were gone down with respect to *S. aureus* where zone of inhibition was reduced by 9 mm. However, the increase in temperature showed no change in zone of inhibition against *S. typhi* and *P. aeruginosa*.

Table 1. *Antimicrobial activity of extracts of P. guajava and A. indica against test bacteria at pH 6.0.*

S. No.	Variable			Antimicrobial activity in terms of diameter of zone of inhibition (mm)			
	Temp (°C)	*P. guajava* (mg/µl)	*A. indica* (mg/µl)	*S. aureus*	*S. typhi*	*E. coli*	*P. aeruginosa*
	35	17.5	17.5	25	12	18	19
	35	25	17.5	18	10	20	23
	35	17.5	25	17	12	22	22
	35	25	25	30	11	26	20
	37	17.5	17.5	18	11	20	19
	37	25	17.5	21	9	23	20
	37	17.5	25	17	10	23	22
	37	25	25	21	11	23	20

At pH 7.5, the maximum inhibition was observed in *S. typhi* where diameter of zone of inhibition was 27 mm by the extract combination of *P. guajava* and *A. indica* at concentration of 25 mg/µl each at temperature 37°C (Table 2). The growth of *E. coli* was most affected negatively by the extract combination of 17.5 mg/µl each at 35°C. However, *P. aeruginosa* was affected most at 35°C by the combined extract of *P. guajava* (25 mg/µl) and *A. indica* (17.5 mg/µl). The antimicrobial activities of both the extracts were severely gone down at pH 9.0 as drastic decreases in zones of inhibition were observed in each bacterial isolate at all combinations and temperatures tested (Table 3). The gentamycin (5 µg/disc) was employed as positive control and the results are presented in Table 4.

Table 2. *Antimicrobial activity of extracts of P. guajava and A. indica against test bacteria at pH 7.5.*

S. No.	Variable			Antimicrobial activity in terms of diameter of zone of inhibition (mm)			
	Temp (°C)	*P. guajava* (mg/µl)	*A. indica* (mg/µl)	*S. aureus*	*S. typhi*	*E. coli*	*P. aeruginosa*
	35	17.5	17.5	14	15	19	15
	35	25	17.5	15	14	17	23
	35	17.5	25	14	14	16	19

	35	25	25	15	14	16	15
	37	17.5	17.5	13	13	17	13
	37	25	17.5	21	17	17	15
	37	17.5	25	15	17	14	18
	37	25	25	27	17	16	13

Results are mentioned as mean of three independent experiments carried out in duplicate

Table 3. *Antimicrobial activity of extracts of P. guajava and A. indica against test bacteria at pH 9.0.*

S. No.	Variable			Antimicrobial activity in terms of diameter of zone of inhibition (mm)			
	Temp (°C)	P. guajava (mg/µl)	A. indica (mg/µl)	S. aureus	S. typhi	E. coli	P. aeruginosa
	35	17.5	17.5	10	9	12	9
	35	17.5	25	11	10	12	9
	35	25	17.5	12	13	12	14
	35	25	25	13	12	13	13
	37	17.5	17.5	12	8	10	9
	37	17.5	25	10	12	12	11
	37	25	17.5	12	13	14	11
	37	25	25	13	13	13	11

Results are mentioned as mean of three independent experiments carried out in duplicate

Table 4. *Antimicrobial activity of gentamycin (positive control) against test bacteria.*

S. No.	pH	Temp. (°C)	Diameter of zone of inhibition (mm)			
			S. aureus	*S. typhi*	*E. coli*	*P. aeruginosa*
1	pH 6.0	35	20	18	25	22
		37	18	21	24	22
2	pH 7.5	35	19	20	23	21
		37	19	22	25	23
3	pH 9.0	35	14	13	15	17
		37	12	15	13	11

Gentamycin concentration: 5 µg/disc

Negative control DMSO was not inhibitory to the tested bacteria.

Discussion

The antimicrobial compounds extracted from plants have great therapeutic potentials against microbes as they can help in ailment without undesirable side effects, which are usually occur with synthetic antimicrobial agents [19]. The ethanolic extracts of leaves of *Psidium guajava* and *Azadirachta indica* were subjected to the screening for antimicrobial activity against four pathogenic bacteria including Gram positive strain *Staphylococcus aureus* and three Gram negative strains *Escherichia coli, Pseudomonas aeruginosa* and *Salmonella typhi*. The antimicrobial activity was tested at three different pH and two different temperatures. The activity of ethanolic extracts of *A. indica* and *P. guajava* was observed most inhibitory at pH 6.0 and temperature 35°C. In present study, the ethanolic extracts of *A. indica* and *P. guajava* have been found possessing antimicrobial activity against test microorganisms. Many plants contain potentially useful substances, which can be used as alternative chemotherapeutic agents. Large varieties of medicinal plants have been screened and many of them have been proven to possess antimicrobial or antifungal activity [20,21]. Therefore, it is a challenge to seek the *in vitro* antimicrobial activity of natural compounds from these ethnomedicinal plants on pathogenic bacteria. A

large number of studies have confirmed that *A. indica* possesses antimicrobial and antifungal activities. In a study, the activity of ethyl acetate extract of Neem (100 µg) was reported comparable to ciprofloxacin (10 µg) against *Campylobacter jejuni* and *Leuconostoc* spp. [22]. In another study, methanolic extract of Neem bark and leaves were found inhibitory to *S. aureus, Klebsiella* and *Pseudomonas* species [23,24]. Okemo and colleagues have estimated the kill kinetics of *S. aureus, E. coli* and *P. aeruginosa* for Neem stem bark extract and found that extract concentration of 0.5 mg/ml significantly reduced *S. aureus* growth within 24 h while extracts with increasing concentrations completely killed the bacteria in lesser time [25].

A study was conducted to evaluate the comparative effect of aqueous, ethanolic and ethyl acetate extracts of Neem leaves on growth of *Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Aspergillus terreus, Candida albicans* and *Microsporum gypseum*. The study found that 20% ethyl acetate extract has the strongest inhibition compared with the activity obtained by the same concentration of aqueous and ethanolic extracts [26]. Goncalves and co-workers conducted a study where they screened the antimicrobial effect of essential oils and methanol, hexane, and ethyl acetate extracts from Guava leaves. They found that the methanol extract showed greatest bacterial inhibition. The essential oil extract showed inhibitory activity against *S. aureus* and *Salmonella* spp. [27]. Vieira and his team have also reported the antimicrobial effect of Guava leaves extracts and found that they inhibited the growth of the *S. aureus* [28]. In a study, Guava leaf extract found to have good antimicrobial activity against nine different strains of *Staphylococcus aureus* [29].

The results of the present study may justify and support the use of extracts of these plants in traditional medicine for the treatment of certain infections. Our results showed an important antimicrobial activity of the *A. indica* and *P. guajava* extracts with clear zones of inhibition against the bacteria tested. Antimicrobial compounds used in different combinations might promote the effectiveness of each agent, with efficacy being achieved using a lower dose of each drug. Pharmacological benefits would increase, with one drug clearing infection from one body system while the other clears it from a different site [30]. In third world countries like Pakistan where contagious diseases are common, it is important to search out and promote plant-derived medicines.

Conclusion

This study highlights the synergistic therapeutic potentials of *Azadirachta indica* and *Psidium guajava* leaves ethanolic extracts *in vitro* against the clinically important strains of Gram-positive and Gram-negative bacteria. The ethanolic leaves extracts of A. indica and *P. guajava* exhibited clear zones of inhibition against the test bacteria indicating that the folk herbal drugs have great potentials as antimicrobial agents. The synergistic effect from the association of different plant extracts against pathogenic bacteria will lead to new choices for the treatment of infectious diseases. It would be advantageous to standardize methods of extraction and *in vitro* testing so that the search could be more systematic and interpretation of results would be facilitated.

Acknowledgement

This article was funded by the Deanship of Scientific Research (DSR), King Abdulaziz University, Jeddah. The authors, therefore, acknowledge with thanks DSR technical and financial support.

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