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A study of antimicrobial activity of Meliaazedarach (Bakayan) plant extracts (aqueous) using pathogenic isolates from patients of Islamabad and Rawalpindi.

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Abstract This study was conducted to determine the in vitro antimicrobial activity of plant extracts of Meliaazedarach. Plants were taken from Islamabad region. Leaves of Meliaazedarach were extracted by "Soxhlet apparatus" and sterilized by Millipore membrane filters. Diffusion and dilution methods were used to determine the antimicrobial activity of aforesaid extracts. In the diffusion method antimicrobial activity of extracts were observed by measuring the sizes of zones of inhibition around the wells containing extracts on Nutrient agar plates. Measures zone sizes were statistically analyzed for significance against Streptomycin (control). In the dilution method the antimicrobial activity was checked in two steps; in the first step extracts were tested qualitatively for antibacterial activity and in the next step these extracts were analyzed quantitatively by standard plate count method against control at 37°C and control at 4°C. In the diffusion method, the leaves extract of Meliaazedarach exhibited significant antimicrobial activity against all 9 bacterial species but in the dilution method, the extract did not show its antibacterial significance against 08 clinically isolated pathogens. Only Pseudomonas growth was inhibited. On these bases future prospects of plant medicines were discussed. In the new era this type of drug research will open the field for scientists to develop safe drugs and industry to serve not only the nation rather the humanity.

Key words: Meliaazedarach, antibacterial activity, growth inhibition, leaf extracts
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INTRODUCTION

Plants have been used during the ages for cure and treatment of diseases since the start of mankind. The seeds, roots, stems and leaves of different herbs, shrubs and trees have been used for centuries to treat the diseases (Moden et al., 1977). The Arabian physicians described the infections of the body and also mentioned several medicinal plant and vegetable substance against diseases such as rabies and hydrophobia in their pharmacopeias or medical formularies (Haq, 1997). The traditional medicines are mostly of plant origin and are still widely used in the regions comprising countries of the third world and China. Plants have also been used to treat other diseases such as diabetes, cardiovascular diseases, thrush, giardiasis, heavy metal poisoning, jaundice, congestion of abdominal and pelvic cavities, reheumatism and scarlet fever etc. (Nadkarni, 1954, said, 1969, Kirtikar et al., 1987, and khan, 1997).

During the past few years, a number of studies have been focused on medicinal evaluation of plants used in traditional medicine. These include examples of Bonafousia species, Croton menthodorum and Heisteria acuminate which possess anti-inflammatory activity and are commonly used in pathologies related to inflammation (Ortega, et al., 1996). Allium sativum not only possesses anti-thrombogenic activity but also contains anti atherogenic effects along with antibacterial, antifungal and anticancer activity (Khan and Basar, 1997). Studies also claimed that some plants, which are already in used as traditional medicine, possess antimicrobial properties against bacteria, Virus, and fungi, and preparation from such plants considered to be effective against diseases of microbial etiology like Hepatitis B & C Tuberculosis, typhoid and diphtheria etc. (Haq, 1997).

When considering antibacterial activity of preparations of plants, studies revealed that plants possess considerable antibacterial activity when compared with modern antibiotics like chloramphenicol and streptomycin (Haq, 1997). Since diseases like typhoid fever and food poisoning are commonly treated with antibiotics like chloramphenicol and ampicillin, the extensive illogical used of these antibiotics have led to the problems of drug resistance (Sanda and Mandell, 1980).

In Pakistan and other third world countries where infectious diseases are prevalent, there is a need to develop some medicines of plant origin against these persisting infectious diseases, which may be comparable to modern medicines and antibiotics. Medicinal plants used in the traditional medicine offer a great reservoir for the discovery of new plants having antimicrobial properties comparable to antibiotics used in modern medicine. Since almost all the antimicrobial agents are being imported and by considering the availability of

medicinal plants in these countries, a lot of foreign exchange may be saved (Haq, 1997). In addition to the cost of treatment is steadily increasing and it is becoming unaffordable by common user. Therefore, the development of therapeutic agents from our own indigenous resources will be of a great help.

In traditional medicine some of the indigenous medicinal plants including Meliaazedarach has been claimed to exert curative effects in the diseases caused by Salmonella species (Nadkarni, 1954; said, 1969).In another study ethnologic fruit extract of Meliaazedarachwas used to check the antibacterial activity against Salmonella typhimurium(Sani et al, 2015). This plant is very common in Rawalpindi and Islamabad and a very little work has been done as yet. Itis commonly used in traditional and homeopathic medicines and people in the countryside of Islamabad and Rawalpindi use the leaves extract of Meliaazedarach to reduce the heat effect in summer despite its extreme bitter taste.

I have studied the in-vitro antibacterial effects of Aqueous leaves extract of Meliaazedarach (Bakayan). Plant extractwas used in aqueous form as it is used in traditional medicine. Antibacterial assays were performed on a variety of clinically isolated Gram positive and Gram negative bacteria.

MATERIALS AND METHODS

This research work was conducted in the bacteriology department of public health division at National Institute of Health, Islamabad.

Plant used in this research

Meliaazedarach plant was used in the study. This plant is very common in Rawalpindi and Islamabad. Its leaves were collected from NIH colony; it was identified by the herbarium of National Agriculture Research Council, Islamabad.

Extraction of active ingredients

Extraction is an important process in the preparation of medicine from plants. This process removes constituents from one phase bringing into contact with a second immiscible liquid phase (Kenneth, 1975). In this experiment "Soxhlet extractor" was used. This extractor comprised of flat bottom flask, chamber to which side arm and siphon tube are attached, along with condenser (Kenneth 1975).

Sterilization of extract

Extracts were sterilized by 0.22 µ membrane filters (Millipore) under positive pressure and kept at 4°C until use (Cheesbrough, 1984).

Media and reagents

Nutrient Agar (Difco.U.K), Nutrient Broth (Difco.U.K), Antibiotic Discs (Oxoid U.K)

Microbial isolates

Microorganisms used in this study were provided by Dr. Muhammad Raheel Afzal Malik, Principal scientific officer and incharge bacteriology laboratory. These organisms were isolated from human blood, urine, throat and pus, in the Bacteriology Laboratory of Public Health Division, National Institute of Health Islamabad. These bacteria were re-identified and their anti-biogram activity was determined. Streptomycin was found most suitable to be used as control (Table 3).

Table 1. Shows the sources of isolated pathogens

Bacteria	Sample
S. aureus	Pus
Strepβ	Throat
E. coli	Urine
K. pneum.	Throat
S. typhi	Blood
S. para-typhi A	Blood
S. para-typhi B	Blood
Pseud aerogi	Urine
Prot. mirabilis	Pus

Anti-microbial activity of plant extracts

In past research on the anti-microbial activity of medicinal plants has been encountering several problems because of the diversity of criteria and techniques employed for testing. The lipophilic properties of some extracts such as oils make it very difficult to use an aqueous medium for the study of anti-microbial activity (Allergini et al., 1973). Among the several methods, which were employed in the plant research, following two conventional method were adapted for this study

Diffusion method and Dilution method

These two methods are being described one by one:

Diffusion method

In this method, microbial culture is inoculated on the surface of agar medium using disk or hole as reservoir of extracts or antibiotics. The same to be tested, present in the reservoir comes into contact with an inoculated medium, and after overnight incubation at 37ºC, the plates are observed for zone of inhibition surrounding the reservoirs. The zone of inhibition is the clear area around the reservoir, shows the inhibition of growth of microorganism by the diffused substance through the agar. The diameter of the clear zone around the reservoir (zone of inhibition) is measured (Rios et al., 1988; Amsterdam, 1988). Well method was used in this study.

Material

Nutrient agar plates, streptomycin as control (15µg/ 100µl) and Crude leaves extract.

Procedure

Dehydrated nutrient agar (23 grams) was mixed with oneliter distilled water and boiled to dissolve the contents of the medium. It issterilized by autoclaving at 121ºC for 20 minutes at 15 Lbs. pressure.When temperature reached between 50 and 60ºC the medium was poured in the petri plates which were already washed and sterilized before the preparation of medium. The medium was poured aseptically in 30ml quantity in each plate; plates were allowed to solidify for 30 minutes and after solidification all plates were incubated at 37ºC for overnight to check for contamination.

Borer, which was comprised off 6mmstainless steel tube attached to the arm of the conical flask and suction pump, which was attached on the mouth of the armed conical flask with a glass tube, assembled the above mentioned components of the borer aseptically. Total 2 holes were cut on the surface of agar medium in each of the 09 plates,those were used in each experiment--- one plates for each bacterium.The holes were marked for Meliaazedarach, and one for Control (Streptomycin).Bacterial cultures were inoculated using cotton swabs after standardization with McFarland standard solution.Each hole was filled with 100ul corresponding product. Plates were kept in the refrigerator for one hour to allow the content of each hole to absorb in the medium.Plates were incubated at 37ºC for 18-20 hours.After incubation the diameter of each zone of inhibition was measured at two different places and the mean value was taken for record.This procedure was repeated 03 times to confirm the size of zone of inhibition and antibacterial effect of extracts on each bacterium used in this study and to evaluate the results (Neycee et al), (Cheesbrough, 1984)

Dilution Method

This method is generally used for quantitative estimation of antimicrobial activities. It is also used in the preliminary screening purpose. In this method turbidity is the indication of growth, which is estimated by colorimetric/spectrophotometric method for quantitative estimation whereas when there is no growth, the medium remains clear, due to anti-microbial activity of samples incorporated in to the medium. (Rios et al., 1988; Vandepitte et al., 1991). The standard plate count method was adopted in the study.

Materials

Media:Nutrient brothtubes and Nutrient Agar plates

Controls: 1) Control 37ºC is the test tube containing 1 ml distilled water instead of plant extract and kept at 37ºC to compare the growth with the treated sample tubes.

2) Control +4ºC: Control +4ºC is the initial load of bacteria used in the test and during the test it was kept at +4ºC to compare the level of growth in the treated sample tubes.

Procedure

Nutrient broth tubes and Nutrient agar plates were prepared and checked for contamination and finally refrigerated until use. 24 hours before the start of experiment the bacterial culture was freshly prepared by inoculating 9ml nutrient broth with 1ml bacterial culture and incubated at 37ºC. McFarland solution was used for standardization purpose.

After overnight incubation at 37ºC the nutrient broth was distributed in 20ml quantity into 100ml flasks. One ml bacterial culture prepared (#2), was inoculated to this flask.

After inoculation medium was distributed in 4ml amount to three tubes. Tubes were marked for Meliaazedarach, Control 37ºC and control +4ºC.

In tubes marked 1ml of extract was added to its corresponding tube. Similarly 1ml distilled water was added to each control tubes. All tubes were incubated at 37ºC for 18-20 hours except the tube marked +4ºC, was kept in the refrigerator at +4ºC.

After overnight incubation turbidity in each tube was checked. Serial dilutions were prepared from each tube upto 10^{-5}. From each dilution 3 plates of Nutrient Agar were inoculated for plate count and incubated for overnight. Colonies on each plate were counted and recorded. Each experiment was repeated three times. Same method was used to test other 8 bacterial cultures.

RESULTS

Physical Characters and pH of Crude Extacts of Meliaazedarach and Berberis vulgaris

The concentrated extracts (5.5 grams) of Meliaazedarach in 50ml distilled water was used to check the color, turbidity and pH (Table 2).

Table 2: Physical features and pH of extracts.

Extracts	Parameters		
	Color	Turbidity	pH
Meliaazedarach Leaves Extract	Dark brown	Turbid	5.78

Diffusion method

In order to show the effectiveness of extracts against each microbe data of 11 experiments has been presented statistically in table 2 and 3 in the form of most commonly used measures of position in statistics. These are mean, Median, and mode (Mould 1998; Zar, 1996). These three values show the complete picture of the effectiveness of extracts against each microorganism in the form of zone size in millimeter (mm). Standard deviation, standard error T value and probability were also calculated, showing significance value of the result.

Table 3: Statistical Analysis of zones of inhibition size (m.m.) in Meliaazedarach leaves extract

Microorganisms	Mean	Median	Mode	Std. Dev.	Std. Err.	t. Test	Probability
E. Coli	23	23	23	0.6742	0.2033	46.38	P<0.001
Pseudomonas	22	22	22	1.1282	0.3402	40.166	P<0.001
S.typhi	25	25	25	0.8312	0.2506	46.483	P<0.001
S.typhi A	23	23	23	1.2136	0.3659	32.357	P<0.001
S.typhi B	21	21	21	1.1201	0.3377	54.327	P<0.001
S.aureus	19	19	19	0.7006	0.2113	16.716	P<0.001
Kl.Pneumoniae	20	20	19	1.0787	0.3252	12.867	P<0.001
Proteus mirabilis	23	23	24	1.4206	0.4283	8.3874	P<0.001
β Streptococci	20	21	21	1.206	0.3636	8.9721	P<0.001

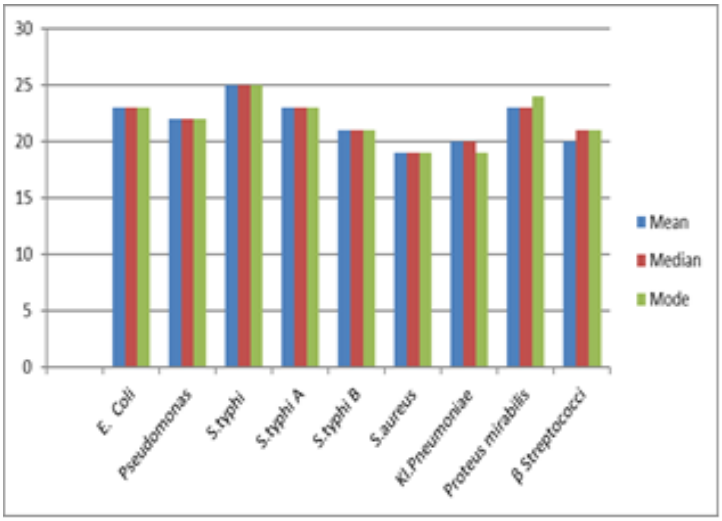

Figure 1: Statistical analysis of zone of inhibition size in Meliaazedarach leaves extract in the form of Mean, Median and Mode.

Table 3: Statistcal analysis of zones of inhibition size (mm), showing the results of control.

Microorganisms	Mean	Median	Mode	.Std.Dev	.Std Err
E. Coli	39	39	39	0.9244	0.2787
Pseudomonas	38	39	39	0.6876	0.2073
S.typhi	40	40	40	0.6742	0.2033
S.typhi A	40	40	40	1.2505	0.377
S.typhi B	40	40	40	0.3015	0.0909
S.aureus	30	31	32	2.0671	0.6232
Kl.Pneumoniae	29	30	30	2.0538	0.6193
Proteus mirabilis	28	29	29	1.3751	0.4146
β Streptococci	28	29	30	2.7002	0.8141

Table 2 and figure 1 show the excellent antimicrobial activity of Mealiaazedarach against 09 clinical isolates. *Salmonella typhi* was found most sensitive microorganism whereas staphylococcus aureus showed least susceptibility to this extract.

The extract of Meliaazedarach showed significant antibacterial activity against each clinically isolated bacterial pathogen although it was in the crude form
(Table 2).Standard deviation and T values show highly significant results (table 2) as the probability is <0.001 in all cases of microorganisms.

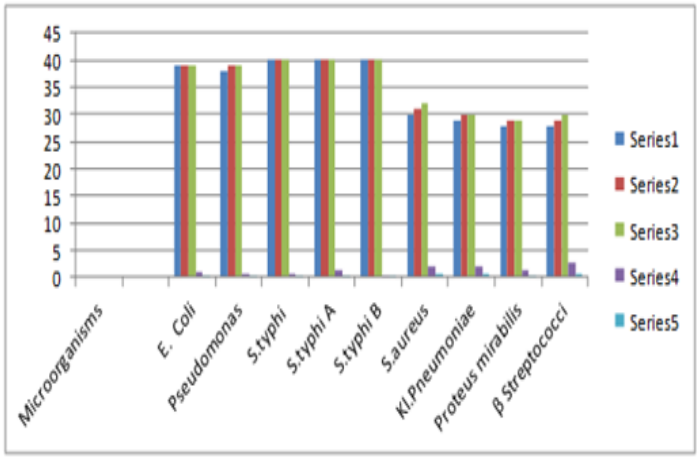

Figure 2: Graph showing the results of Control as given in table 3

Dilution method

In this method all the selected microorganisms were tested separately and the sensitivity of each microorganism against the plant extract was checked thrice. But it was observed that the extract of Meliaazedarach inhibited the growth of *Pseudomonas aeroginosa* only and in other cases the extract promoted the growth of all eight bacteria.

DISCUSSION

In the diffusion method Meliaazedarach leaves extract showed its affectivity against various pathogens. *Salmonella typhi* was found most sensitive microorganism against the antibacterial effects of the extract. Salmonella paratyphi A, E.coli and Proteus mirabilis were shared the number position on the basis of their susceptibility to this extract, whereas the antibacterial activity of the extract was found least against Staphylococcus aureus.

If results of Meliaazedarach leaves extract are compared with the results of streptomycin (control) it seems encouraging in the sense that the antibacterial activity of this extract was above the level of that standard on which most of the commonly used antibiotics are considered as sensitive; although it was used in the crude form and may also contain growth promoting factors.

When results of diffusion and dilution methods are compared, it is observed that in the diffusion method Meliaazedarach extract was found more effective against all pathogens. But in the dilution method it inhibited the growth of *Pseudomonas aeroginosa* only. The reason might be the solubility and diffusebility of active compounds present in the extracts which may either be growth promoting or inhibitory to microbes or sensitivity of microbes to these compounds (Rios et al., 1988).

CONCLUSION

In the new era this type of drug research will open the field for scientists to develop safe drugs from such types of plants, exist in the country and industry to serve not only the nation rather humanity.

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