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COMPARATIVE ANALYSIS OF ANTIMICROBIAL POTENTIAL OF BITTER AND NON BITTER LEAVES OF UNUSUAL *AZADIRECTA INDICA*

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ABSTRACT

Medicinal plants are part of humans since the dawn of civilization. In recent years, multiple drug resistance has developed due to indiscriminate use of synthetic drugs. This force the need to screen medicinal plants for their novel compounds, as plants based drugs are biodegradable, harmless and with lesser side effects. We selected the *Azadiracta indica* plant from the locality of Hingoli district having both bitter and non bitter leaves on the same plant. The alcoholic and aqueous extracts of *Azadiracta indica* plant collected were evaluated for antimicrobial activity by Disc diffusion method different organisms. To determine inhibitory the inhibitory effect the activity of *Azadiracta indica* against different organisms was tested by serial broth dilution method and was expressed by minimum inhibitory concentration (MIC).

KEY WORDS: *Azadiracta indica,* aqueous extract, alcoholic extract, Antimicrobial activity.

1. INTRODUCTION

Medicinal plants are gifts of nature to cure vast numbers of diseases among human beings. It has well known since ancient times that plants and spices have antimicrobial activity .There has been a considerable interest to use plants and spices for the elimination of microorganisms. According to World Health Organization more than 80% of the worlds population relies on traditional medicine for there primary healthcare requirements. Utilization of herbal medicines in Asia represents a long history of human interaction with the surroundings. Plants used in traditional medicinal contain a wide range of ingredients that

can be use to treat chronic as well as communicable diseases. How to use the plants against different illnesses may be expected to have accumulated in areas where the use of plants is still of great importance.[1]

Now a day's multiple drug resistance has developed due to the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases. Antibiotics are sometimes associated with the adverse effects on the host like hypersensitivity and allergic reactions. This situation forced scientist to search for new antimicrobial substances. Due to the alarming Incidence of antibiotic resistance in bacteria of medical importance, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases from medicinal plants. There are several reports on the antimicrobial activity of different herbal extracts in different regions of the world.[2]

The *Azadiracta indica* tree is noted for its drought resistance. Normally it flourishes in areas with sub-humid conditions, with an annual rainfall 400–1,200 millimetre (16–47 in). *Azadiracta indica* can grow in many different types of soil, but it nurture best on well drained deep and sandy soils. It can tolerate high to very high temperatures and does not tolerate temperature below 4 °C (39 °F).[3] Shown to possess insecticidal, nematicidal, fungicidal ,anti;inflammatory, antiworm, immunostimulating, antiviral, antiseptic, anti inflammatory, antiworm, antimalaria, antiulcer, antipeptic, antripyretic and antilibido properties.[4] Its extract has been used in poultry to disperse glandular tumors and ulcer while paste in skin disease like eczema and leprosy and scabiasis.[5] The extract has also been used in jaundice (hepatitis) and liver complaints. Fruit juice and ripe fruits have been used as purgative, astringent, tonic, eyesore treatment, demulcent and emollient.[6] Oil has been used as pesticide, as contraceptive, as antiseptic in tooth paste and soap.[7]

2. MATERIALS AND METHODS

Collection & Processing of Samples

Fresh leaves sample of unusual *Azadirachta indica* plant with better and non better leaves on same plant body were collected from locality of district Hingoli in Maharashtra State of India on 5th December 2014. The samples were then finally washed with running tap water to remove soil and adhering dust particles. Only green leaves were included. For the present study the bitter and non bitter leaves were collected separately and dried individually in shadow.

Chemicals

The reagents used in this study were ethanol, glacial acetic acid, H_2SO_4 50% (v/v), Peptone (Himedia), Yeast extract (Himedia), Nutrient Agar (Himedia), MH Agar (Himedia), Gentamycie (500 mg) (Himedia)All the chemicals were of analytical grade.

Microbial cultures

Following microorganisms were used as test organism for evolution of antimicrobial activity. These were obtained from National Collection of Industrial Micro-organisms (NCIM). The collected cultures of bacteria were sub cultured on nutrient agar (HiMedia) slants respectively and stored at 4°C until required for study.

1. *Enterobactor cloclae (NCIM2164)*
2. *Pseumonas fluerescenceC2(NCIM2059)*
3. *Bacillus subilities(NCIM2921)*
4. *Lactobacillus Plantorum (NCIM2085)*
5. *Staphylococcus aureus (*NCIM 2901)
6. *Bacillus megaterium(NCIM2087)*
7. *Klebshiella pneumoniae(NCIM2720)*
8. *Esherichia.coli (NCIM2803)*
9. *Pseudomonas(NCIM2004)*
10. *Salmonella typhi* (NCIM2501)

1. Extracts

1. Preparation of *Azadirachta indica* aqueous extract-*Azadirachta indica* aqueous extract was prepared by mixing 15.0gm of dry powder of *Azadirachta indica* leaves with 100ml of sterile distilled water in a round bottom flask with infrequent shaking. The extract was then filtered through a muslin cloth for coarse residue and finally through Whatman paper and kept in an airtight amber colored container.[8]

2.Preparation of Azadirachta indica ethanolic extract-Azadirachta indica extract was prepared by macerating 15.0gm of dry powder of *Azadirachta indica* leaves with 100ml of 70%(w/v) ethyl alcohol for a week in a round bottom flask with occasional shaking. The flask was kept in the dim light to avoid effect of light on the active ingredients of *Azadirachta indica.* The extract was then filtered through Whatman paper and kept in an airtight amber colored container.[8]

Antimicrobial Sensitivity Test

1. Activation of culture –inoculums preparation

Antibacterial activity of the crude extracts in different solvents was tested by disc diffusion assay. Mueller Hinton (HiMedia) was used as the bacteriological medium. Medium was prepared and poured 20 ml each in sterilized Petri plates of 9 mm diameter and allowed to solidify. Bacterial cultures grown in nutrient broth and on agar slants were used. Bacterial suspension was prepared aseptically from 10 ml of saline (0.085 g NaCl in 10 ml Distilled water) under laminar. The plates, cultured with microbial suspension (100-150 µl) by spread plate technique. The bacterial cultures were maintained on Nutrient agar pH 7.0± 0.2) at 4^0C temperature respectively. Media and growth conditions like temperature (37 ^{0}C for bacteria), and incubation period (24-48 hrs for bacteria) used for culturing these strains were as prescribed by Agarkar Research Institute (ARI), Pune and National Collection of Industrial Microorganisms (NCIM); NCL, Pune.[9]

2. Well diffusion method

The 20ml of sterile MH agar was poured into sterile petriplates, after solidification, 100ul of fresh culture of bacteria were swabbed at respective plates. The wells were made over the agar plates using sterile gel puncher of each aqueous and alcoholic *Azadirachta indica* extract for both bitter and non bitter leaves extract. The plates were incubated for 24 hours at 37°c.After incubation the diameter of zones of inhibition was measured in cm.[9]

3. Measurement of zone

The anti bacterial activity was evaluated by measuring the diameter of the inhibition zone formed around the well. The experiments were performed in triplicate and the mean diameter of the zone of inhibition was calculated.[9]

4. Minimum inhibitory concentration (MIC)

The respective bacterial strain from the stock was revived by plating on MH Agar medium. After overnight incubation at 37°c, isolated colonies were selected and the identities of the organisms were confirmed. By obtaining the serial dilution, the concentration of *Azadirachta indica* powder was achieved as 100%, 50%, 25% and 12.5% respectively. The colonies were streaked on MH agar plates and discs deeped in different concentrations were placed on plates. The plates were then incubated for 24hours at 37°c. After the incubation, the MIC values were determined by visual inspection of plates. In this experiment the last disc with clear zone of inhibition was considered to be without any growth and taken as MIC value. A

similar procedure of serial dilution was carried out to test the antimicrobial activity of *Azadirachta indica* with non bitter leaves. Same procedure of serial dilution as mentioned above was carried out to test antimicrobial activity of the alcoholic *Azadirachta indica* having bitter and non bitter leaves extract.[7]

5) Determining the growth curve of bacterial cells exposed to different concentration of extract

To study the effect of alcoholic extract on growth of Enterobacter cloacae (NCIM 2164) culture were streak on MH agar and next day single colony was picked and grown in MH broth until the growth was reach up to 2x 10^2. Then one ml of this culture was inoculated in MH broth containing minimum inhibitory concentration of better and non better extract that is 25 % and culture without extract is used as control and after every one hour optical density was recorded.[10]

6) Assaying the effect of extract on protein leakage from bacterial cell membranes

Protein leakage from bacterial cells was detected using Bradford's protein assay the concentration of extract was adjusted to 25% and the concentration of bacterial cells was 105 CFU/ml. Each flask containing culture was incubated at 37^0 C for 6 hr. 1ml of culture sample was obtained from each flask. The sample was centrifuge at 4^0 C for 30 min at 300xg and supernatant was used for estimations protein concentration. The supernatant was treated with Bradford's reagent and the O.D. was measured at 595 nm.[11, 12]

RESULT AND DISCUSSION

For thousands of years, there has been target interest in biologically active compounds, isolated from plant species for the eradication of pathogenic micro-organisms, because of the resistance that micro-organisms have built against antibiotics.[13] Result obtained in antibacterial activity of extracts of *Azadirecta Indica (bitter and non bitter) is* summarized in table 1. In the present study in an all total 10 microbial culture including gram positive and negative bacteria have been tested against *Azadirecta Indica* extracts. For antimicrobial assay it was slotted into four parts; first is bitter aqueous extracts, non bitter aqueous extract, bitter alcoholic extract and non bitter alcoholic extract. These all four showed antimicrobial activity against *Bacillus megaterium (NCIM2087). Klebshiella pneumoniae (NCIM2720),* Enterobacter *cloacae (NCIM 2164)* and *pseudomonas.* Amongst these only alcoholic extract of bitter and non bitter shows antimicrobial activity against *lactobacillus Plantarum (NCIM2085).*

The zone of inhibition (ZOI) for bitter and non bitter alcoholic extract has been shown in table no.1. In present study it was found that non bitter extract shows more antimicrobial activity than bitter extract. The results of present study indicate that *non bitter Azadirachta indica* is more effective against *Enterobacter cloacae (NCIM 2164)* than bitter.

Minimum inhibitory concentration (MIC)

In the present study the Enterobacter cloacae (NCIM 2164) was resistant upto 12.5% concentration of bitter and 25% concentration non bitter alcoholic extracts. Hence 25% is considered as minimum inhibitory concentration of *Azadirachta indica* for *Enterobacter cloacae* (NCIM 2164).

Growth curve of bacterial cells treated with different concentration of extract

Result obtained in effect of growth on *Enterobacter cloacae* (NCIM 2164) by extracts of *Azadirachta indica* (bitter and non bitter) is summarized in fig. 1. The control shows the maximum growth while bitter and non bitter extracts affect the growth of *Enterobacter cloacae* (NCIM 2164). As compared to bitter the non bitter inhibit the remarkable growth of *Enterobacter cloacae (NCIM 2164).* The result of this study is that non bitter *Azadirachta indica* is more effective than bitter against *Enterobacter cloacae (NCIM 2164*).

Effect of non bitter extract on protein leakage from bacterial cell membranes

It was found that non bitter extract could enhance protein leakage by increasing the membrane permeability of *Enterobacter cloacae (NCIM 2164).*Initially, protein leakage from the membranes of *Enterobacter cloacae (NCIM 2164*) cells treated with extract was almost same as that on the cells of control group .At 6 hrs of after incubation, protein leakage from cells treated with extract was considerably increased ;however there was no significant change in the amount of protein leakage from cells in the control group(fig. 2).

The literature indicates that medicinal plants have secondary compounds[13] in same way *Azadirachta indica* plant is also have secondary compounds like nimbin, nibidin and other compouns which are responsible for their various activities.[14-16] Which type of secondary compound present or absent in these non bitter leaves which shows maximum activity against *Enterobacter cloacae (NCIM 2164)* is still to discover.

Table 1: Evaluation of Zone of inhibition (ZOI) of extract against micro organisms. Zone of inhibitions are after subtracting 3mm disc diameter from the total diameter

Name of Microorganism tested	Aqueous bitter	Alcoholic bitter	Aqueous non bitter	Alcoholic non bitter	Standard Gentamycine
Enterobacter cloacae (NCIM 2164)	4 mm	6 mm	5 mm	**10 mm**	8mm
Pseumonas .Fluerescence C2(NCIM2059)	-	-	-	-	6 mm
Bacillus subtilis(NCIM2921)	-	-	-	-	5 mm
Lactobacillus. Plantorum (NCIM2085)	-	2 mm	-	4 mm	5 mm
Staphylococcus aureous (NCIM 2901)	-	-	-	-	3 mm
Bacillus megaterium (NCIM2087)	3 mm	**7 mm**	4 mm	**8 mm**	4 mm
Klebshiella. pneumonae	4 mm	**6 mm**	5 mm	**7 mm**	3 mm
Escherichia coli (NCIM2803)	-	-	-	-	5 mm
Pseumonas aeroginosa (NCIM2004)	4 mm	**5 mm**	5 mm	**8 mm**	6 mm
Salmonella typhi (NCIM2501)	-	-	-	-	5 mm

Table 2: MIC of Enterobacter cloacae (NCIM 2164)

Extract of *Azadirecta Indica*	**100%**	**50%**	**25%**	**12.5%**
Alcoholic bitter	**S**	**S**	**R**	**R**
Alcoholic non bitter	**S**	**S**	**S**	**R**

R-Resistant

S-Sensitive

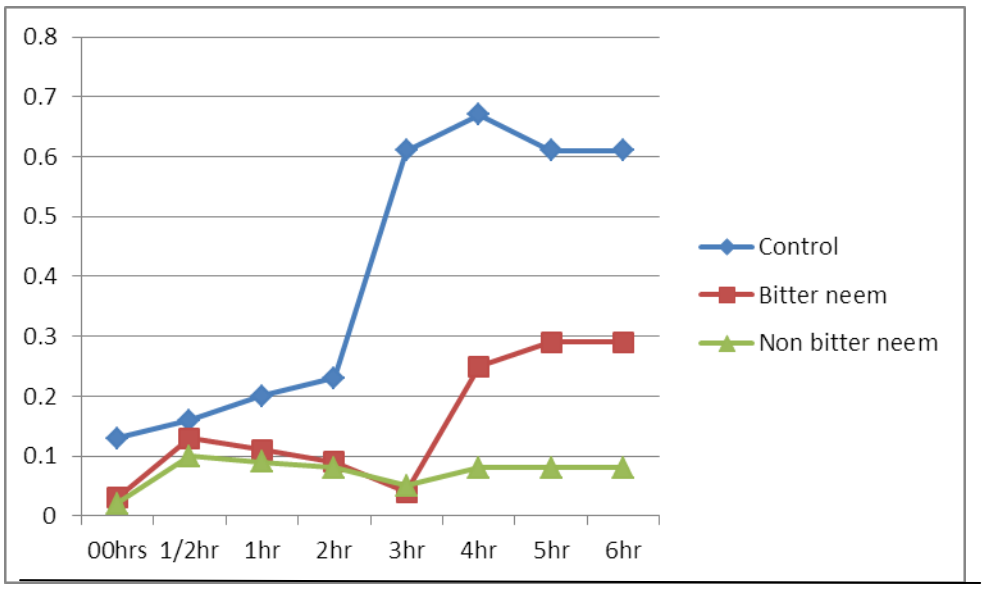

Fig 1-Effect of alcoholic extract (25%) on growth of Enterobacter cloacae (NCIM 2164).

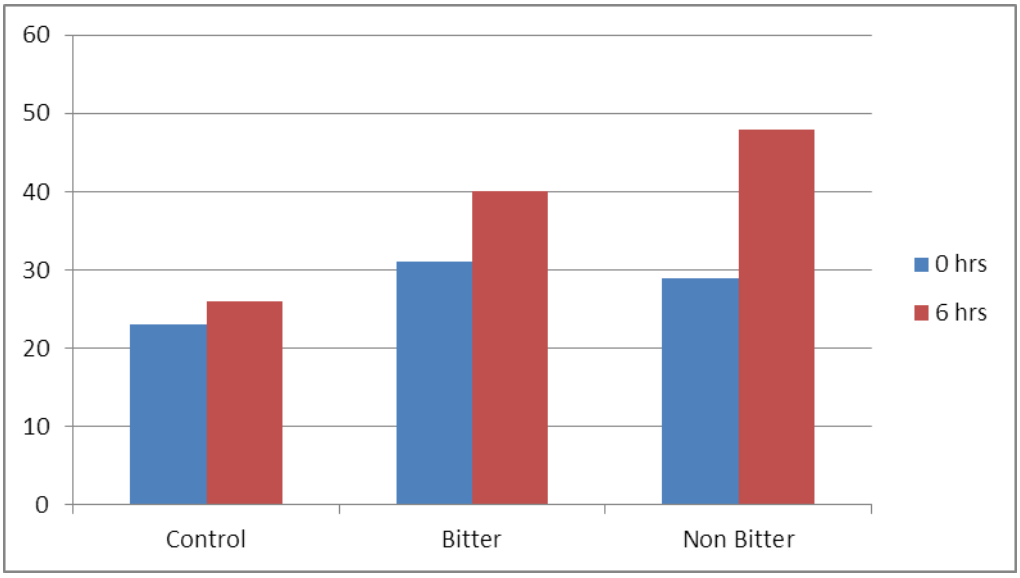

Fig. 2. Leakage of protein(ug/ml) from Enterobacter cloacae(NCIM 2164) cells exposed to alcoholic bitter and non bitter extract of *Azadirecta Indica*. Bacterial cells was treated at the concentration of 25%, and the control was not treated. Non bitter extract was significantly different from control group after 6 hrs of exposure.

CONCLUSION

Result obtained in antibacterial activity of extracts of *Azadirachta indica (bitter and non bitter)* is summarized in table 1. In the present study in an all total 10 microbial culture including gram positive and negative bacteria have been tested against *Azadirachta indica* extracts. After MIC and effect of alcoholic extract on growth of Enterobacter cloacae (NCIM 2164) it is found that some compound is present in non bitter *Azadirachta indica* to have significant antimicrobial activity and therefore can be used as natural antimicrobial agent for the treatment of several infectious diseases caused by *Enterobacter cloacae (NCIM 2164)* and further to develop antibiotics. This can be now concluded that after the laboratory experiments non bitter *Azadirachta indica* can be used as natural therapeutic drug against *Enterobacter cloacae (NCIM* 2164). Finding out the genetic mutation that occurs in this unusual plant which causes changes in bitter leaves and it convert into non bitter should be the future direction for researchers.

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