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EVALUATION OF ANTIBACTERIAL AND ANTICANDIDIAL EFFICACY OF AQUEOUS AND ALCOHOLIC EXTRACT OF NEEM (*AZADIRACHTA INDICA*) AN *IN VITRO* STUDY

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ABSTRACT

Medicinal plants are part and parcel of humans since the dawn of civilization. In recent years, multiple drug resistance has developed due to indiscriminate use of synthetic drugs. This drives the need to screen medicinal plants for novel bioactive compounds as plant based drugs are biodegradable, safe and have fewer side effects.

Neem (*Azadirachta indica)* is perhaps the most commonly used traditional medicinal plant of India. Almost all parts of the plant are endowed with medicinal properties. Several pharmacological activities and medicinal applications of various parts of Neem have been documented in the ancient literature. Teeth and their supporting structures are subject to infections by Streptococcus species, a number of facultative anaerobes like Enterococcus faecalis, and opportunistic pathogens like Candida albicans. Literature shows that *Neem* is a powerful agent that inhibits the increase and establishment of microorganisms that cause infectious diseases in the oral cavity.

In the present study we have evaluated the antimicrobial potential of Neem leaf aqueous and alcohol extracts. To determine the inhibitory effect of *Azadirachta indica* (aqueous and alcoholic extract of neem) on Streptococcus mutans, Enterococcus faecalis and Candida albicans. The activity of *Azadirachta indica* against Candida albicans, Streptococcus mutans and Enterococcus faecalis was tested by serial broth dilution method and was expressed by minimum inhibitory concentration (MIC). The minimum inhibitory concentration (MIC) of the aqueous neem extract to all the organisms was 7.5%. The MIC of the alcoholic neem extract for *E. faecalis, S.mutans, C. albicans* were 1.88%, 7.5%, and 3.75% respectively.

KEYWORDS: Aqueous Neem extract, alcoholic neem extract, *E. faecalis, S.mutans, C. Albicans,* serial broth dilution, MIC.

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INTRODUCTION

Medicinal plants are part and parcel of humans since the dawn of civilization. In India they form the backbone of several indigenous traditional systems of medicine. Pharmacological studies have acknowledged the value of medicinal plants as potential source of bioactive compounds[1]. Medicinal plants are a rich source of novel drugs that form the ingredients in traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates, bioactive principles and lead compounds in synthetic drugs[2]. In recent years, multiple drug resistance in both human and plant pathogens has developed due to indiscriminate use of synthetic drugs.

This drives the need to screen medicinal plants for novel bioactive compounds as plant based drugs are biodegradable, safe and have fewer side effects[1].

Azadirachta indica is well known in India and its neighboring countries as one of the most versatile medicinal plants having a wide spectrum of biological activity. *A. indica* and *M. azedarach* are two closely related species of Meliaceae. The former is popularly known as Indian Neem (margosa tree) or Indian lilac, and the latter as the Persian lilac[3,4].

Neem (*Azadirachta indica* A. Juss) is perhaps the most commonly used traditional medicinal plant of India. Almost all parts of the plant are endowed with medicinal properties and have been used as traditional medicine or household remedies against various human ailments, from antiquity. In this era, Neem is considered as a valuable source of unique natural products for development of medicines against various diseases[4,5] . Only crude extracts of different parts of Neem have been used as traditional medicine for treatment of various diseases. Neem has been extensively used in Ayurveda, Unani, Homoeopathic and Siddha medicine and has become a cynosure of modern medicine[6] .

Dental caries and diseases of the periodontium are among the most common afflictions of oral diseases of mankind[7]. Teeth and their supporting structures, are subjected to infection by *Streptococcus* bacteria that causes cavities and gingivitis[8].

Primary endodontic infections caused by oral microorganisms, are usually opportunistic pathogens that may invade a root canal containing necrotic tissue and establish an infectious process. Enterococcus faecalis, a facultative anaerobic gram-positive coccus, is the most common Enterococcus species cultured from non healing endodontic cases. It is usually isolated in pure culture or as a major component of the flora of previously root filled teeth with chronic apical periodontitis[9].

Oral candidiasis is the most common oral fungal infection in humans, primarily caused by C. albicans. Candida are important opportunistic pathogens owing to the high frequency with which they cause infections in immune deficient individuals such as cancer patients, those receiving broad spectrum antibiotics, HIV-infected and AIDS patients[10].

Mechanical therapies, including scaling, root planning and surgery, are aimed at improving clinical conditions by lowering the microbial load either by physical removal of plaque or by radical alteration of the subgingival habitat. Antimicrobial approaches, including the use of systemically and locally administered antibiotics, directly target subgingival species residing in the plaque biofilm or in the adjacent epithelial tissues lining the periodontal pocket.

The deployment of natural substances for use in dentistry is just gaining momentum. Research in this field endeavours to analyse the chemical properties and the workings of these compounds. Since the use of antibacterial agents may be restricted by side effects, great importance is given to natural alternatives for the prevention or decrease of microorganism adhesion[11].

The aim of the current study is to evaluate the antimicrobial effect of Neem (*Azadirachta indica)* leaf aqueous and alcohol extract as a potential agent in the inhibition of Streptococcus mutans, Enterococcus faecalis and Candida albicans.

MATERIALS AND METHODS

A standard procedure for performing the MIC test was followed[12]. The standard strains of the organisms used in this study were Streptococcus mutans – ATCC 25175, Enterococcus faecalis – ATCC 35550, Candida albicans ATCC 2091. Brain Heart Infusion broth (BHI), Sabouraud dextrose broth, Neem leaf aqueous and alcohol extracts, sterile MIC tubes and micropipettes were the other armamentarium used. Statistical analysis of the results was done by Fisher Exact test of significance[13].

Neem leaf powder of 96-97% purity was procured from M/s Shanbag Ayur Products, Yallapur, Uttar Kannada district, Karnataka.

Preparation of neem aqueous extract

Neem aqueous extract was prepared by mixing 15.0 g of dry powder of neem leaves with 100 ml of sterile distilled water in a round bottom flask with occasional shaking. The extract was then filtered through a muslin cloth for coarse residue and finally through Whatman No. 1 filters paper and kept in an airtight amber coloured container.

Preparation of neem ethanolic extract

Neem extract was prepared by macerating 15.0 g of dry powder of neem leaves with 100 ml of 70% (w/v) ethyl alcohol for a week in a round bottom flask with occasional shaking. The flask was kept in the dark to avoid effect of light on the active ingredients of the neem. The extract was then filtered through a muslin cloth for coarse residue and finally through Whatman No.1 filters paper and kept in an airtight amber coloured container.

A preliminary pilot study was conducted to test the activity of various concentrations of aqueous/alcoholic neem extract on Streptococcus mutans, Enterococcus faecalis and Candida albicans using the agar diffusion method. The various concentrations of aqueous/alcoholic neem extract used were 15%, 10%, 7.5%, 5%, 2.5% and

1%. Various volumes of aqueous/alcoholic neem extract measuring 75µl, 50µl, 25µl, 10µl and 5µl were tested, using agar diffusion method.

Interpretation of diffusion results was done by noting the presence or absence of zone of inhibition around the wells. A zone of inhibition of 12 mm or above was considered as sensitive and a zone of less than 12mm was considered as resistant.

Minimum inhibitory concentration (MIC) determination for the aqueous neem extract

Procedure: Revival of the organisms – The respective bacterial and candidial strains from the stock was revived by plating on blood agar medium. After overnight incubation at 37^0 C, isolated colonies were selected and the identities of the organisms were confirmed. Isolated colonies were transferred to sterile BHI broth and Sabouraud dextrose broth for the bacteria and candida strain respectively and once again incubated overnight. The growth concentration was adjusted to 10^5organisms / ml by using 0.5 McFarland's turbidity standard.

An aqueous solution of 15% concentration was prepared from the neem powder as the stock solution. Two hundred µl of the BHI broth was added in each of ten MIC tubes per bacterial strain. For the Candida strain 200µl of the Sabouraud dextrose broth was added in each of ten MIC tube. In the first MIC tube containing 200 µl broth, 200 µl of stock was added. After mixing well, 200 µl was transferred to the second MIC tube. This was continued till the last (10^{th}) tube. From the last tube 200 µl final solution was discarded. By following this serial dilution, the concentration of the neem powder was achieved as the following 15%, 7.5%, 3.75%, 1.88%, 0.94%, 0.47%, 0.23%, 0.12%, 0.06%, 0.03% respectively.

To each of the ten such prepared MIC tubes with varying concentrations, 200 µl of the earlier prepared strain of S.mutans was added such that the final volume per tube was 400 µl. The procedure was repeated for the E.faecalis and the Candida albicans strain. The tubes were then incubated for 24 hours at 35^0 C.

After the incubation, the MIC values were determined by visual inspection of the tubes. With each batch of tests, positive and negative controls were put up. Positive control containing broth plus bacterial / candidial strain showed turbidity and negative control containing broth only appeared clear. In each series of tubes, the last tube with clear supernatant was considered to be without any growth and taken as MIC value. Turbidity in the MIC tube indicated growth of the bacteria / candidial strain implying that the organisms were resistant to the aqueous neem extract**.**

Minimum inhibitory concentration (MIC) determination for the alcoholic neem extract

A solution of 15% concentration was prepared as the stock solution. The working concentration for the neem extract was achieved as the following 15%, 7.5%, 3.75%, 1.88%, 0.94%, 0.47%, 0.23%, 0.12%, 0.06% and 0.03% respectively. A similar procedure of serial dilution as mentioned above was carried out to test the antimicrobial, anticandidial activity of the alcoholic neem extract.

RESULTS

Table 1: *E. faecalis* showed sensitivity to aqueous extract of neem in concentrations of 15%, 7.5% and demonstrated resistance to concentrations of 3.75%, 1.88%, 0.94%, 0.47%, 0.23%, 0.12%, 0.06% and 0.03%. *S.mutans* showed sensitivity to neem aqueous extract in concentrations of 15%, 7.5%, and resistance to concentrations of 3.75%, 1.88%, 0.94%, 0.47%, 0.23%, 0.12%, 0.06% and 0.03%. *C. albicans* showed sensitivity to neem aqueous extract in concentrations of 15%, 7.5%, and resistance to concentrations of 3.75%, 1.88%, 0.94%, 0.47%, 0.23%, 0.12%, 0.06% and 0.03% . Hence the MIC of neem aqueous extract for *E. faecalis,* was established at 7.5%, *S.mutans* at 7.5%, and *C. albicans* at 7.5 %

Table 2: *E. faecalis* showed sensitivity to alcoholic neem extract in concentrations of 15%, 7.5%, 3.75%, and 1.88% and demonstrated resistance to concentrations of 0.94%, 0.47%, 0.23%, 0.12%, 0.06% and 0.03% . *S.mutans* showed sensitivity to alcoholic neem extract in concentrations of 15%, 7.5%, and resistance to concentrations of 3.75%, 1.88%, 0.94%, 0.47%, 0.23%, 0.12%, 0.06% and 0.03%. *C. albicans* showed sensitivity to alcoholic neem extract in concentrations of 15%, 7.5%, 3.75% and resistance to concentrations of 1.88%, 0.94%, 0.47%, 0.23%, 0.12%, 0.06% and 0.03% . Hence the MIC of alcoholic neem extract for *E. faecalis,* was established at 1.88%, *S.mutans* at 7.5%, and *C. albicans* at 3.75%.

The results show that the MIC of aqueous neem extract for E faecalis was 7.5% as against 1.88% for the alcoholic neem extract. This difference however was not statistically significant (p= 0.243). The MIC of both the aqueous and alcoholic extract of neem for S mutans was 7.5%. The MIC of aqueous neem extract for C albicans was 7.5% as compared to the alcoholic neem extract being at 3.75% .The difference was not statistically significant (p=0.348).

DISCUSSION

Initiation of periodontal disease occurs through the accumulation of a thin film of bacteria on the surface of the teeth called plaque. The disease is sustained by

various pre-disposing factors or risk factors like anatomic defects, host response, genetic predisposition of the patient and so on.

In the agar diffusion test, there was microbial growth observed around all the wells with all concentrations and varying volumes implying the resistance of the organisms to the aqueous and alcoholic neem extract. This could have been because of failure of neem to diffuse effectively through the agar medium. With this observation we decided to use the serial broth dilution method to determine the MIC of aqueous and alcoholic neem extract.

Rajshekharan *et al*[14] concluded that leaf extracts of *A.indica* exhibited significant anti-bacterial activity against all the test microorganisms. However, inhibitory activities of the leaf extracts were both organism and solvent dependent. The leaf extracts limited the growth of both Gram positive and Gram negative bacterial species tested. Among the different extracts used in the study, ethanolic and dichloromethane leaf extracts of *A. indica* were found to be more active towards the bacterial species used in the study. Further, the aqueous leaf extract was moderately active. In our study the MIC for S.mutans for both the aqueous and ethanolic neem extracts were 7.5 %. The difference in these two studies could be because the organisms tested were different and a disc diffusion method was used by Rajshekharan to establish relative antibacterial activity whereas we tested and even established the MIC levels of the antibacterial activity of both aqueous and ethanolic extracts of neem leaf powder.

Chemical investigation on the products of the neem tree was extensively undertaken in the middle of the twentieth century. Since the early report by Siddiqui[15] in 1942 on the isolation of nimbin, the first bitter compound isolated from neem oil, more than 135 compounds have been isolated from different parts of neem and several reviews have also been published on the chemistry and structural diversity of these compounds[16-24]. The compounds have been divided into two major classes: isoprenoids and others[23]. The isoprenoids include diterpenoids and triterpenoids containing protomeliacins, limonoids, azadirone and its derivatives, gedunin and its derivatives, vilasinin type of compounds and Csecomeliacins such as nimbin, salanin and azadirachtin. The nonisoprenoids include proteins (amino acids) and carbohydrates (polysaccharides), sulphurous compounds, polyphenolics such as flavonoids and their glycosides, dihydrochalcone, coumarin and tannins, aliphatic compounds, etc. The details of the chemistry of various compounds falling under these groups have already been reviewed[22,23]

Oil from the leaves, seeds and bark possesses a wide spectrum of antibacterial action against Gram-negative and Gram-positive microorganisms, including *M. tuberculosis* and streptomycin resistant strains[25]. *In vitro*, it inhibits *Vibrio cholerae*, *Klebsiella pneumoniae*, *M. tuberculosis* and *M. pyogenes*[26]. Antimicrobial effects of neem extract have been demonstrated against *Streptococcus mutans* and *S.* faecalis[27]. Data in literature suggest that certain species such as S.mutans require hard surfaces for sustained colonization although they may be detected in dentate subjects at low levels on the soft tissues. Studies have suggested that S. mutans essentially disappears from the oral cavity when all the teeth are extracted and reappears if hard surfaces are provided in the form of full dentures[28].

Wolinsky and co-workers[29] suggested that neem stick extract can reduce the ability of some streptococci to colonize tooth surfaces. Prashant and co-workers[30] demonstrated that Neem stick extract produced the maximum zone of inhibition on Streptococcus mutans at 50% concentration. Even at 5% concentration neem extract showed some inhibition of growth for all the four species of organisms - Streptococcus mutans, Streptococcus salivarius, Streptococcus mitis, and Streptococcus sanguis which are involved in the development of dental caries. Vanka *et al*[31] concluded that while Streptococcus mutans was inhibited by Neem mouthwashes used for a period of two months, with or without alcohol as well as chlorhexidine, lactobacillus growth was inhibited by chlorhexidine alone.

Our study demonstrated good antibacterial activity against S. mutans by both the aqueous and alcoholic extract demonstrating an MIC at 7.5%.

Bokhora and co-workers[32] concluded that neem leaf extract has a significant antimicrobial effect against *E. Faecalis* (derived from infected root canal samples) and *C.Albicans.* Annie Pritima and Selvaraj Pandian[33] established antibacterial activity of neem leaf in different extracts against bacteria cultured from vaginal swabs and concluded that for ethanol extract - E. faecalis, E. Coli and P. mirabilis showed the maximum zone of inhibition, for methanol extract - highest activity was observed for E. coli, E. faecalis and P. mirabilis. For acetone extract - maximum zone of inhibition was seen for both E. faecalis and E. coli. For aqueous extract - E. Faecalis, S. aureus, B. cereus and K.pneumoniae showed moderate activity. On comparison it was observed that E. coli and E. faecalis were highly susceptible to neem extracts of all solvents.

In our study the MIC of aqueous neem extract for E.faecalis was 7.5 % as against 1.88% for the alcoholic neem extract. This difference was however not

statistically significant (p=0.243) which corroborates with the above studies.

Extracts of neem leaf, neem oil and seed kernels are effective against certain human fungi, including *Trichophyton*, *Epidermophyton*, *Microsporum*, *Trichosporon*, *Geotricum* and *Candida*[34]. High antimycotic activity with extracts of different parts of neem has already been reported[35]. Azadirachta indica extract was tested in vitro on strains of Candida albicans 12A and 156B. Neem leaf aqueous extract did not inhibit the growth of any strain in concentrations as from 0.1 g/ml as growth of yeast colonies occurred in all tested dilutions. Neem leaves have a potential anti-adhesive effect on the samples studied in vitro. An anti-adhesive mechanism of action by Azadirachta indica was proposed based on the results observed[36]. Neem oil exhibited antifungal against *Candida albicans* (ATCC 10231) because of the sulphur compound contents[37].

In our study the MIC of aqueous neem extract for C. albicans was 7.5% as compared to the alcoholic neem extract being at 3.75%. However this difference was also not statistically significant (p=0.348). But the fact that anticandidial activity was demonstrated even at such low concentrations of 7.5% and 3.75% is excellent.

CONCLUSION

Aqueous and alcoholic extract of neem leaf showed significant antibacterial activity against S.mutans and E.faecalis and significant antifungal activity against C albicans. There was no statistical difference between the efficacies of alcoholic over aqueous neem extract. However, further studies with larger samples containing clinical isolates of various periodontal pathogens may shed more light on the efficacy of these extracts and their probable use in clinical settings.

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Table 1: Shows the results of Aqueous neem extract

Microorganisms	Concentration of aqueous neem extract									
	15%	7.5%	3.75%	1.88%	0.94%	0.47%	0.23%	0.12%	0.06%	0.03%
E. faecalis	S	S	R	R	R	R	R	R	R	R
S.mutans	S	S	R	R	R	R	R	R	R	R
C. albicans	S	S	R	R	R	R	R	R	R	R

S= SENSITIVE R= RESISTANT

Table 2: Shows the results of Alcoholic neem extract

Microorganisms	Concentration of alcoholic neem extract									
	15%	7.5%	3.75%	1.88%	0.94%	0.47%	0.23%	0.12%	0.06%	0.03%
E. faecalis	S	S	S	S	R	R	R	R	R	R
S.mutans	S	S	R	R	R	R	R	R	R	R
C. albicans	S	S	S	R	R	R	R	R	R	R

S= SENSITIVE R= RESISTANT

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