Volume-3, Issue-7, July-2014 • ISSN No 2277 - 8160

Research Paper Pharma

Pharmacognostic Effects of *Achyranthes aspera* roots and Ocimum sanctum roots and Leaves on *Streptococcus mutans* Causing Dental Caries.

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ABSTRACT

Dental caries is one of the most important problems in public health because of its ubiquitousness in civilized populations.S. mutans is known as the causative bacteria in the formation of dental plaque and dental caries. The acid producing S. mutans inhabiting the mouth causes damage by dissolving tooth structures in the presence of fermentable carbohydrates such as sucrose, fructose, and glucose (Kleinberg I, 2002).This study was done to check the use of chemicals present in a very well known toothpaste X and to provide the knowledge of the use of herbal antimicrobial i.e.Achyranthes aspera roots and Ocimum sanctum roots and leaves. Bacterial sample was collected from saliva and dental plaque. Estimation of Streptococcus mutans was done using sheep blood agar medium, Mitis salivarius, bacitracin agar medium and gram staining. Confirmatory tests like catalase and carbohydrate fermentation were also performed. MIC was done using serial dilution method and brain heart infusion was taken as supplement media. Bacterial isolates were identified as positive Gram stained, catalase negative, spherical or ovoid shape, negative growth at pH 9.6, alpha haemolytic, Bacitracin resistant and sucrose positive. Aqueous and alcoholic extract of Achyranthes aspera rootsandOcimum sanctum roots and leaves was prepared atconcentration of 0-50% and 1-10% respectively to observe MIC. Similarly aqueous and alcoholic extract of Toothpaste X was also prepared at all concentrations but found no MIC at any concentration (Graph 3). Aqueous and alcoholic extract of Ocimum roots also did not show MIC at any concentration. Comparable work on both aqueous and alcoholic extract of Achyranthes aspera rootsandOcimum sanctum leaves showed that alcoholic extract of Ocimum sanctum leaves is most effective at 6%.

KEYWORDS : Fermentable, Prevalence, Ubiquitousness, Haemolytic.

1. Introduction

It has been well documented that traditional medicinal plants confer considerable antibacterial activity against various microorganisms (Jonathan et al.,2000). Many plants were reported to inhibit the growth of many oral bacteria (Pack et al., 1998), particularly Streptococcus mutans and control plaque and thus prevent caries have been investigated (Jagtap and Karkera,2000; Margan et al., 2001). Effective prevention of caries' infections can be achieved by mechanical removal of dental plaque by proper tooth brushing and flossing. However, the majority of the population, particularly aged individuals, may not perform mechanical plaque removal sufficiently, and thus antimicrobial mouth rinses such as triclosan and chlorhexidine may be used to limit these two plaque-related oral infections (Baca P.et al.,2009). These chemical agents used in the form of either dentifrices or mouth rinses may have undesirable side effects such as tooth staining, taste alteration and development of hypersensitivity reactions (Chang YC et al.,2001 and Beaudouin E et al.,2004).

Plants have traditionally provided a source of hope for novel drug compounds, as plant herbal mixtures have made large contributions to human health and well-being. They are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found in vitro to have antimicrobial properties (Cowan, 1999; Lewis and Ausubel, 2006). The chemical constituents present in plants are a part of the physiological functions of living flora and hence they are believed to have better compatibility with the human body (Kamboj, 2000). They have stood the test of time for their safety, efficacy, cultural acceptability and lesser side effects. Plant derived medicines have been the first line of defense in maintaining health and combating diseases. The herbal products today symbolise safety in contrast to the synthetics that are regarded as unsafe to human and environment (Cragg et al., 1997). Through this study the approach is to compare the medicinal properties of two plant parts i.e roots of Achyranths aspera and roots and leaves of Ocimum sanctum with each other and with Toothpaste X.

One of the many plants which are being evaluated for their therapeutic efficacies is Achyranthes aspera which is commonly known as Latjeera (Hindi) & Rough Chaff tree (English). It is an erect or procumbent, annual or perennial herb, 1-2m in height, often with a woody base, commonly found as a weed of waysides, on roadsides (Jitendra B et al.,2006,). Traditionally, the plant is used in asthma and cough. It is pungent, antiphlegmatic, antiperiodic, diuretic, purgative and laxative, useful in oedema, dropsy and piles, boils and eruptions of skin etc. Crushed plant is boiled in water and is used in pneumonia. Infusion of the root is a mild astringent in bowel complaints (K.M.Nadkarni,2009).

A fresh piece of root is used as tooth brush. Paste of the roots in water is used in ophthalmia and opacities of the cornea. S.K. Sharma et al. (2009) from the ethanolic extracts of the roots isolated a new aliphatic acid and identified as n-hexacos-14-enoic acid from the roots of A. aspera. This compound is reported for the first time from any natural and synthetic source.

According to Cowan M M 1999, Tulsi, scientifically known as Ocimum sanctum, is a time-tested premier medicinal herb. . It is a herb that is bestowed with enormous antimicrobial substances and is used to treat a variety of illnesses ranging from diabetes mellitus, arthritis, bronchitis, skin diseases, etc. (Prakash P and Gupta N 2005, Bhat M et al.,2008 and Viyoch J et al., 2006) Recent studies have also demonstrated significant anticancer properties of Ocimum sanctum (Magesh V et al.,2009) Hence, it is also termed as the queen of herbs or the mother medicine of nature. Eugenol (Ihydroxy- 2-methoxy-4-allylbenzene), the active constituent present in Ocimum sanctum, perhaps is largely responsible for the therapeutic potential of Tulsi (Prakash P and Gupta N 2005). The other important constituents include ursolic acid and carvacrol. The antimicrobial activity of Tulsi can be attributed to these constituents.

2. Material and method

2.1 Method of sample collection:

Sample was collected from saliva and dental plaque .Gingival plaque was taken by sterile tooth pick and was collected from interproximal surfaces of posterior teeth and 1.0 ml of normal saline was added to it in test tube. Salivary sample was made pure by centrifugation.

2.2 Estimation of Streptococcus mutans:

1. By sheep blood agar medium-Blood Agar is a bacterial growth medium that can distinguish normal from pathogenic bacteria

based on the effect of bacterial hemolytic exotoxins on red blood cells. Blood agar (BAP) is a differential growth medium which microbiologists use to distinguish clinically significant bacteria from throat and sputum culture. It produced white or grey, circular, sometimes rather hard and coherent and tending to adhere to surface of agar. Itcontains: Nacl-5.0 g, Agar-10.0 gm, special peptone-23.0gm, starch-1 gm, distilled water-1000 ml and 5% defibrinated sheep blood at Ph 7.3.

2. MitisSalivarius bacitracin Agar (MSB Agar)-It produced rough, heaped colonies, often with beads , droplets on or around the colonies while some formed smooth or mucoid colonies (Fig.2). It contains: Mitissalivarius agar base-100gm, Sucrose-200gm, Potassium tellurite-1 ml of (1% in DW), Bracitracin solution -1 ml and distilled water 1000ml.
3. Gram staining- In this procedure bacterial cells are stained with a basic dye(crystal violet), treated with iodine-KI mixture to fix the stain, washed with alcohol and counter stained with safranin. Some bacteria retains the stain during the subsequent steps hence they are Gram positive bacteria while some decolorize by the organic solvent and hence they are gram negative bacteria.

2.3 Biochemical tests for Identification of Streptococcus mutans

(a) Catalase test-

A few drops of 1% solution of hydrogen peroxide was placed directly on the bacterial colony.Rapid effervescence indicates production of molecular oxygen and a positive test (Fig.4).While absence of effervescence shows catalase negative test (Fig.3).

(b) Carbohydrate catabolism (oxidation/ fermentation)-

Saccharolytic micro-organisms degrade glucose either fermentatively or oxidatively.The end products of fermentation are strong mixed acids that can be detected in a fermentation test medium. Some tubes of medium were inoculated with overnight grown cultures. To one of the tubes of each pair, a layer of liquid paraffin was added to a depth of about 1 cm, incubated at 37ºC and examined for 5 days.

(c) Growth at pH 9.6-5% -

Sheep blood agar plates of pH 9.6 were used. Plating was done and incubated at 37˚C for 48 hours. After incubation the plates were examined for appearance of growth.

2.4 Preparation of plant extract:

To compare the effect of plant extract on Streptococcus mutans following two types of extracts were prepared:-

Aqueous extract:

For aqueous extract ,leavesofAchyranthes aspera and Ocimum sanctum were collected and soaked in distilled water, blotted dry, made into a slurry through blending, and then strained or filtered. The filterate can be centrifuged multiple times for clarification. Similarly aqueous solution of toothpaste X was made.

Alcoholic extract:

For alcoholic extract, plant parts and Toothpaste X are dried, ground to a fine texture, and then soaked in ethanol for extended periods. The slurry is then filtered and washed, after which it may be dried under reduced pressure and re- dissolved in the alcohol to a determined concentration.

2.5 Minimum inhibitory concentration:

MIC is defined as the lowest concentration of a compound/extract/ drug that completely inhibits the growth of the microorganism in 24h(Anejaet al.,2009). The MIC for the aqueous and ethanolic extract was determined by using Brain heart infusion which is employed for the propagation of fastidious pathogenic cocci and other organisms associated with blood culture work and allied pathological investigations. Brain-Heart Infusion Broth can be supplemented with antibiotics, varying amounts of sodium chloride, yeast extract, and serum to provide a rich medium for bacteria, yeasts and pathogenic fungi. (Atlas R M, 1993)

Aqueous and alcoholic extract of both plant materials was prepared and serially diluted at known concentrations. Brain heart infusion medium prepared using 37gm/1000L was also serially diluted according to extract concentration. Prepared media and extract were then inoculated with Streptococcus mutans and incubated at 37˚C for 24 hours. Along with the sample test tubes blank was also prepared to calculate and know minimum bacterial concentration. After 24 hours optical density of bacteria was calculated by substracting the value of blank from sample with the help of spectrophotometer at 660nm (Table 2,3 and 4).

Fig.1 Colonies of Streptococcus mutans On blood agar medium.

Fig.2 Colonies of Streptococcus mutans On MSB medium.

Fig. 3 Catalase Negative test.

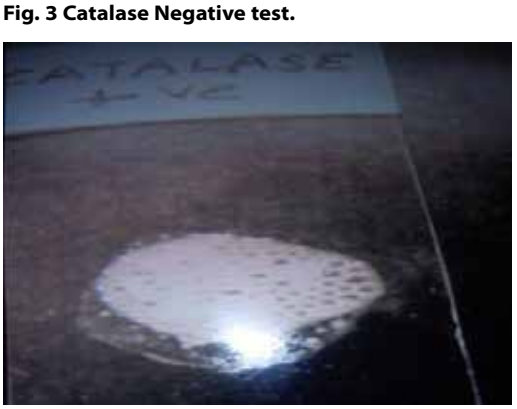


Fig.4 Catalase positive test.

3.Result
Identification criteria of isolates:

Table- 1 Bacterial isolates were identified through staining followed by morphological and biochemical tests:

Characteristics	S.mutans
Shape	Spherical or ovoid
Gram staining	Positive
Arrangement	In pair
Catalase test	Negative (Fig.3)
Growth at pH 9.6	Negative
Haemolysis	Alpha (Fig.1)
Carbohydrate Fermentation	
Sucrose	Positive

When aqueous and alcoholic extracts of both Achyranthes aspera roots and Ocimum sanctum leaves at concentration of 0%, 10 %, 15 %, 20%, 25%, 30%, 35%, 40%, 45%.50% and 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10% respectively was applied against Streptococcus mutans using Serial broth dilution method to calculate minimum bacterial concentration with the help of spectrophotometer (Table 2,3 and 4) it was observed that alcoholic extract ofOcimum sanctum-leaves at 6% is most effective than all Achyranthes aspera roots extract (Graph 1 and 2)

Table- 2 Comparison of Alcoholic and Aqueous extract of Ocimum sanctum leaves at different concentrations to observe minimal optical density.

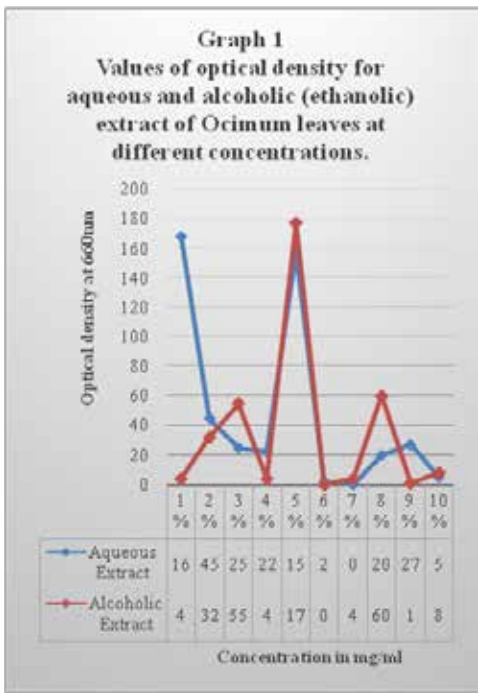
Alcoholic (ethanol) extract (mg/ml)	Optical density (at 660nm)
6% 7%	00 04
Aqueous extract (mg/ml) 6% 7%	 02 00

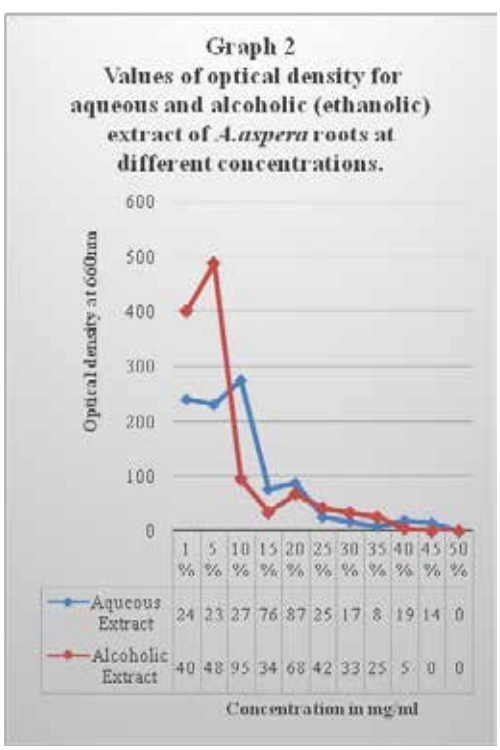
Table- 3 Comparison of Alcoholic and aqueous extract of Achyranthes asperaroots at different concentrations to observe minimal optical density.

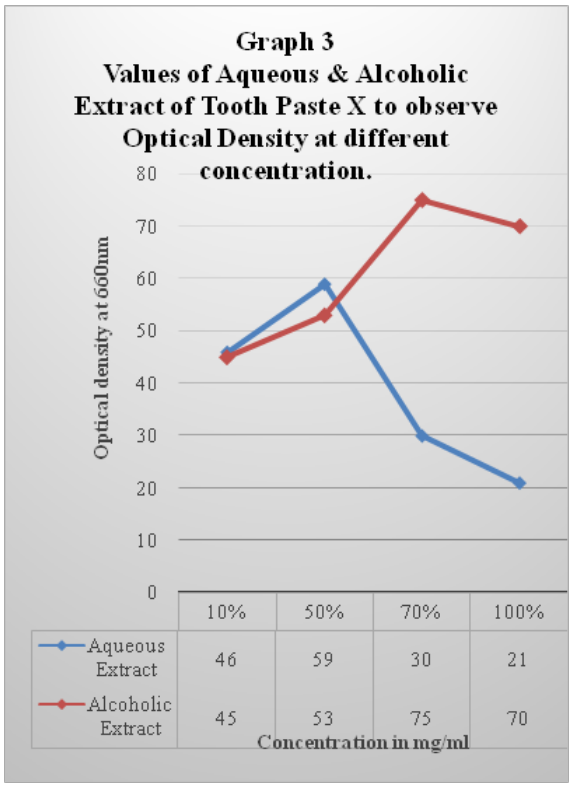
Alcoholic (ethanol) extract (mg/ml)	Optical density (at 660nm)
0% 15% 25% 35% 45% 50%	612 34 42 25 00 00
Aqueous extract (mg/ml) 0% 15% 25% 35% 45% 50%	 383 76 25 08 14 00

Table- 4 Comparison of Alcoholic and aqueous extract of Ocimum sanctum and Achyranthes asperaroots to observe minimal bacterial concentration.

Alcoholic (ethanol) extract (mg/ml)	Optical density(at 660nm)
Ocimum sanctum 6% Achyranthes aspera 45% 50%	 00 00 00
Aqueous extract (mg/ml)	Optical density (at 660nm)
Ocimum sanctum 7% Punicagranatum 50%	 00 00







4.Discussion

The long and venerable history of the use of plants to improve dental health and promote oral hygiene has been known since antiquity. Cutting of root, stem or twigs of trees and shrubs have served as traditional toothbrush commonly called chewing sticks (Almas K 2002). The Bible offers descriptions of approximately 30 healing plants. Even in the book of Exodus of the Bible we find description of plants. Indeed, frankincense and myrrh probably enjoyed their status of great worth due to their medicinal properties. Thus natural cures and remedies are known to give long term benefits to the patients. Not only to these age old remedies cure the pain, they treat the root of the infection and improve the patien's oral hygiene. Clinical microbiologists have two reasons to be interested in the topic of antimicrobial plant extracts. First, it is very likely that these phytochemicals will find their way into the arsenal of antimicrobial drugs prescribed by physicians; several are already being tested in humans. Second, the public is becoming increasingly aware of problems with the over prescription and misuse of traditional antibiotics and third, time to time resistant microorganisms against antibiotics are increasing.Plants have traditionally provided a source of hope for novel drug compounds, as plant herbal mixtures have made large contributions to human health and well-being. They are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found in vitro to have antimicrobial properties (Cowan, 1999; Lewis and Ausubel, 2006). The medicinal actions of plants are unique to a particular plant species or group, consistent with the concept that the combination of secondary products in a particular plant is taxonomically distinct (Parekh et al., 2005). They have stood the test of time for their safety, efficacy, cultural acceptability and lesser side effects. Plant derived medicines have been the first line of defense in maintaining health and combating diseases. The herbal products today symbolise safety in contrast to the synthetics that are regarded as unsafe to human and environment (Cragg et al., 1997). An important characteristic of plant extracts and their components is their hydrophobicity, which enable them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable (Sikkema et al., 1994). Ahmad et al.(1998) screened medicinal plants to detectantimicrobial activity and clearly demonstrated that alcohol is a better solvent as compared to aqueous and hexane. Similarly water was not found to be the most effective solvent for extracting the active compounds from plants as compared to hexane and methane (Shale et al.,1999). InTulsi leaves are quite effective in treating common oral infections. Also few leaves chewed help in maintaining oral hygiene. Carracrol and tetpene are the antibacterial agents present in this plant (Agarwal P et al., 2010). The roots of Achyranthes aspera contained Ecdysterone and Oleanolic acid. These phytochemicals act as spermicidal (D.Paul et al., 2010), antipyretic (N.G.Sutar et al., 2008) and as a cardiovascular agent (N.C. Neogi et al., 1970). The toothpaste used for the comparison contains natural products as main ingredient and also some chemicals along with it like calcium carbonate, Sorbital, Silica, Sodium Lauryl sulphate, Flavour, Cellulose gum, Carrageenan, Sodium silicate, PVM/MA Copolymer, Sodium sachharin and Benzoate, CI 77891, Triclosan etc. Some may suggest that it is probably advisable to use mouthwash at least an hour after brushing with toothpaste when the toothpaste contains SLS , since the anionic compounds in the SLS toothpaste can deactivate cationic agents present in the mouthrinse (Rosenberg, Mel 2002). In this study it is observed that alcoholic extract of Ocimum sanctum leaves at 6% concentration is most effective and its aqueous extract is effective at 7% (Graph 1 and Table 2). Like Ocimum sanctumleaves its roots were also testedagainst S.mutans but they were not effective against it any concentration. Extract ofAchyranthes aspera roots were also prepared starting from 0% to 50% but like O. sanctum leaves this extract was not effective at lower concentrations. Continuing the work it was observed that 50% of aqueous extract was effective against Streptococcus mutans and its alcoholic extract was found to be effective against Streptococcus mutans at 45% and 50% concentration both (Table 3 and Graph-2). Working on all concentrations it was observed that toothpaste X was not effective against S.mutans even at single concentration may be due to presence of chemicals in it (Graph-3).Although alcoholic extract of Ocimum sanctum leaves is most effective among all extracts against Streptococcus mutans(Table-4).

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