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EVALUATION OF ANTIMICROBIAL POTENTIAL OF LEAVES OF *ANACARDIUM ACCIDENTALE* L. ON ORAL CARIOGENIC AND PERIODONTAL PATHOGENS AND A FUNGAL ORGANISM *CANDIDA ALBICANS*

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ABSTRACT

Aim: Due to the emergence of multi-drug resistant organisms, there is a need for the development of new antimicrobials more importantly from natural sources as this delays the development of resistance. The antimicrobial capabilities of plant extracts derived from the leaves of the cashew plant, *Anacardium occidentale L.*, on oral cariogenic and periodontal pathogens and a fungal organism *Candida albicans* were studied. **Methods:** The leaves of *Anacardium occidentale* were collected and different solvents were used for the preparation of the extract namely petroleum ether, dichloro methane, ethyl acetate, methanol and aqueous solvent. The antibacterial potential was evaluated using the agar well diffusion assay. **Results:** The methanol extracts of *Anacardium occidentale* at 500mg/ml concentration showed maximum zone of inhibition against *Prevotella intermedia* (14mm) followed by *Streptococcus mitis* and *Lactobacillus species* (12mm each) and *Streptococcus mutans* (10mm). The *Candida albicans* was found to be resistant against all the different extracts studied. The antimicrobial activities of leaves of *Anacardium occidentale* against the test organisms are promising. **Conclusion:** Exploring the biologically active compounds and understanding their nature and mechanisms of action are open to investigation which can be the basis for the development of new antimicrobial agents from *Anacardium occidentale*.

KEYWORDS: *Anacardium occidentale*, Antimicrobial, Cariogenic, Periodontal.

INTRODUCTION

Drug resistance to pathogenic bacteria is increasingly encountered all over the world in recent years.[1,2] Antibiotics are also associated with adverse effects including allergic reactions, hypersensitivity, depletion of beneficial gut and immunosuppresssion.[3] Therefore, there is a need to search for alternative antimicrobial agents in the treatment of various microbial diseases. Herbal medicines have been used in medical practices to treat various diseases since ancient time. Plant sources have made a great contribution towards maintaining human health and are also associated with fewer side effects when compared to allopathic medicines.[4] According to WHO (1993), 80% of the world's population is dependent on the traditional medicine and these therapies mainly involve the use of plant extracts or their active constituents.[5] Therefore, studies on medicinal plants to explore their antimicrobial properties have gained immense importance in recent years.

Anacardium occidentale (*A. occidentale*) *L*. belonging to the Family *Anacardiaceae*, is a tree of the tropics which grows on relatively dry soil in nature but on cultivation grows well in the tropical rain forest.[6] It is grown for the nuts (true fruit) having an exclusive fine taste and a commercial importance of its own. The edible cashew apple (the thick receptacle or 'false fruit' to which the cashew nut or true fruit is attached) has high nutritive values such as high vitamin C content, minerals such as calcium, phosphorus and iron.[7,8]

It is a multipurpose tree with great economic and medicinal value. Its leaves, stems and bark extracts are used extensively for the treatment of various diseases. It has been reported to possess anti-diabetic, anti-bacterial, anti-inflammatory and anti-ulcerogenic properties.[9] The leaves have found their importance in the treatment of eczema, psoriasis, scrofula, dyspepsia, genital problems, and venereal diseases, as well as for impotence, bronchitis, cough, intestinal colic, leishmaniasis, and syphilis-related skin disorders.[10] The cashew apple is reported to possess antimicrobial,[11,12] antioxidant,[13,14] antitumor,[15] antimutagenic properties.[16]

The leaves of *A. occidentale* are used traditionally for toothaches and gum problems.[17] Therefore, the present study was carried out to know the antimicrobial properties of leaves of *A. occidentale* against common oral cariogenic and periodontal pathogens.

MATERIAL AND METHODS

Preparation of plant extract

Preparation of petroleum ether extract

The leaves of *A. occidentale* were collected locally and authenticated. After collection, it was washed with distilled water to remove dirt. The barks were cut into very small pieces and dried at room temperature under shade for 21 days. The barks were ground into a coarse powder with the help of a suitable grinder and the powder was then stored in an airtight container and stored in a cool, dark and dry place till they were used for solvent extraction. 100 grams of the plant powder was soaked in 1000ml of petroleum ether and stirred at room temperature for 24 hours. The mixture was filtered through Whatman filter paper and filtrate was concentrated to dryness at 45^0C on a rotary evaporator. The extract prepared was refrigerated to be used as such for further studies. The residual precipitate was extracted further as described below.

Preparation of dichloromethane extract

Residue obtained after extraction with petroleum ether was further soaked in dichloromethane and stirred at room temperature for 24 hours. The mixture was filtered through Whatman filter paper and filtrate was concentrated to dryness at 45^0C on a rotary evaporator. The extract prepared was refrigerated to be used as such for further studies. The residual precipitate was extracted further as described below.

Preparation of ethyl acetate extract

Residue obtained after extraction with dichloromethane was further soaked in ethyl acetate and stirred at room temperature for 24 hours. The mixture was filtered through Whatman filter paper and filtrate was concentrated to dryness at 45^0C on a rotary evaporator. The extract prepared was refrigerated to be used as such for further studies. The residual precipitate was extracted further as described below.

Preparation of Methanol extract

Residue obtained after extraction with ethyl acetate was further soaked in methanol and stirred at room temperature for 24 hours. The mixture was filtered through Whatman filter paper and filtrate was concentrated to dryness at 45^0C on a rotary evaporator. The extract used was refrigerated to be used as such for further studies. The residual precipitate was extracted further as described below.

Preparation of Aqueous extract

Residue obtained after extraction with methanol was further soaked in distilled water and stirred at room temperature for 24 hours. The mixture was filtered through Whatman filter paper and filtrate was concentrated to dryness at 45^0C on a rotary evaporator. The extract prepared was refrigerated to be used as such for further studies.

Collection of test organisms

Test organisms were collected from carious cavities of affected teeth by scraping soft caries using excavator and from periodontal pockets using paper points. After collection the paper points were dropped into 20 ml of brain heart infusion broth (BHI broth) which was used as transport media.

The plates were incubated at 37^0C for 48 hrs aerobically and anaerobically (media streaked in duplicate – one for aerobic and the other for anaerobic). The different types of colonies were picked up, isolated and subcultured onto sheep blood agar plates for further identification. Colonies of different test organisms were identified by colony morphology, gram staining, catalase test, pigment production, aerotolerance and sugar fermentation tests. The organisms isolated from the samples included *Streptococcus mutans (S. mutans)*, *Streptococcus salivarius (S. salivarius)*, *Streptococcus mitis (S. mitis)*, *Lactobacillus species*, *Prevotella intermedia (P. intermedia)*, and *Candida albicans (C. albicans)*.These organisms were preserved for studies by repeated subculturing on blood agar slants and maintained in deep freezer at -80^0C. For antibacterial activity studies, fresh subcultures are done in BHI broth and used as inocula.

Determination of antimicrobial activity

The extracts of leaves of *A. occidentale* prepared using different solvents were dissolved in DMSO, in the concentration of 5G/10 ml and then filtered using whatman filter paper no.1. The dissolved extract was then diluted using dimethyl sulfoxide (DMSO) to obtain a concentration of 500 mg/ml, 250 mg/ml and 125 mg/ml.

The susceptibility of the test bacteria to plant extracts was determined using an agar well diffusion assay on 5% sheep blood agar plates. Fresh 24 hour old broth cultures of bacteria were adjusted to 0.5 McFarland turbidity (1-2 x 10^6CFU mL) and spread evenly over the entire surface of the agar plates using a sterile cotton swab. The plates were allowed to air-dry for approximately 10 min following which 5 wells (6 mm holes) were cut into the agar using sterile steel borer.

Individual wells were filled with plant extracts (50 µL). Three wells were filled with the different concentrations of extract 500mg/ml, 250 mg/ml and 125 mg/ml. 2.5% Sodium hypochlorite and DMSO were pipetted into the other two wells and used as positive and negative/solvent control respectively. The plates were incubated at 37ºC for 48 hours period. For each microorganism tested, zones of inhibition of growth were examined, and the diameter of each zone was measured and recorded. Each concentration included triplicates and the results are average of three independent experiments.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The minimum inhibitory concentration (MIC) of the different extracts of *A. occidentale* was determined by broth dilution method. Sterile Brain Heart Infusion broth, 1 ml was taken in test tubes to which 10 microlitres of the fresh bacterial inoculums were added. Then the extract was added in the concentrations of 125, 60.5, 30.25, 15.13, 7.56 mg/ml to each tube. Tube containing only bacterial inoculums served as growth control and sterile BHI broth served as negative control. The tubes were incubated at 37 degree centigrade for 24 hours. The tubes were checked for turbidity and the lowest dilution showing turbidity was taken as MIC. Subcultures were done on Blood agar from each of the tubes and the plates incubated for 24 hours at 37^0C. The lowest dilution that did not grow any colony was taken as MBC.

RESULTS

Sodium hypochlorite which was used as positive control was effective against all test strains of bacteria and *Candida albicans*. DMSO did not show any zone of inhibition against test organisms which acted as negative control. All the examined extracts of *A. occidentale* showed varying degrees of antibacterial activities against the tested pathogens.

At a concentration of 125 mg/ml, the 5 different extracts of *A. occidentale* did not show zone of inhibition against any of tested bacteria and fungi. The methanol extract of *A. occidentale* at 250mg/ml concentration showed an inhibition zone of 8 mm each for *P. intermedia* and *Lactobacillus species*. The results of antimicrobial activity of A. occidentale at 500mg/ml concentration are given in table-1. The methanol extracts of *A. occidentale* showed maximum zone of inhibition against *P. intermedia* (14mm) followed by *S. mitis* and *Lactobacillus species* (12mm each) and *S. mutans* (10mm). It did not show zone of inhibition against *S. salivarius* and *C. albicans.* The petroleum extract showed maximum activity against *P. intermedia* and *Lactobacillus species* (10mm). It was also effective against *S. mitis* and *S. mutans* with zone of inhibition of 8mm each. The ethyl acetate exhibited maximum activity against *S. mitis* and *Lactobacillus species* (10mm) followed by *S. mutans* (8mm). The aqueous extract of *A. occidentale* was effective against *Lactobacillus species* (8mm). The dichloromethane extract failed to demonstrate any antibacterial and antifungal activity against tested pathogens.

The methanol extract of *A. occidentale* showed maximum zone of inhibition against cariogenic and periodontal pathogens. Therefore MIC and MBC were evaluated for this extract. The results are given in table-2. The MIC and MBC value for *S. mitis* was 60.5mg/ml and 125mg/ml respectively. The *Lactobacillus* and *P. intermedia* species

showed MIC and MBC values of 15.13mg/ml and 60.5mg/ml respectively. The MIC and MBC values for all other organisms were more than the highest concentration tested as all the concentrations tested showed growth in MIC tubes.

Table 1: Showing the zone of inhibition in mm for *Anacardium occidentale* extract at 500mg/ml concentration.

Anacardium occidentale	*S. mutans*	*S. mitis*	*S. salivarius*	*Lactobacillus species.*	*Prevotella spies.*	*Candida*
Petroleum ether	8	8	6	10	10	6
Dichloro methane	6	6	6	6	6	6
Ethyl acetate	8	10	6	10	6	6
Methanol	10	12	6	12	14	6
Aqueous	6	6	6	8	6	6
Positive control (Sodium hypochlorite)	16	18	12	20	16	10

Table 2: Showing MIC and MBC value of methanolic extract of *Anacardium occidentale.*

Anacardium occidentale	MIC (mg/ml)	MBC (mg/ml)
S. mitis	60.5	125
Lactobacillus species	15.13	60.5
P. intermedia	15.13	60.5
S. mutans	>125	>125
S. salivarius	>125	>125
Candida albicans	>125	>125

DISCUSSION

Dental caries and periodontal diseases are considered to be the two most common oral health problems across the world. Dental caries is a localized, progressive demineralization of the hard tissues of the crown and root surfaces of teeth caused by acidogenic and aciduric Gram-positive bacteria, primarily the *S. mutans, Lactobacilli* and *Actinomycetes.* Acids produced by bacteria ferment dietary carbohydrates leading to the demineralization of tooth surfaces.[18,19] Periodontal disease is a group of disease involving the gingival, periodontal ligament and alveolar bone and are mainly due to certain bacteria present in subgingival plaque. *Actinobacillus actinomycetemcomitans (A. actinomycetemcomitans), Porphyromonas gingivalis (P. gingivalis*), *Prevotella intermedia (P. intermedia),* and *Tannerella forsythensis (T. forsythensis)* present in the subgingival plaque lay an important role in the in the formation of the periodontal pocket, connective tissue destruction, and alveolar bone resorption and subsequent development of periodontitis.[20] Oral candidiasis is one of the most common human infections of fungal nature. It has been described as an opportunistic infection, frequently involved with oral microbiota alteration, systemic diseases, and reduction of the host immunity. Among the strains implicate in oral candidiasis development, *C. albicans* is the most prevalent and highest pathogenic microorganism.[21,22] As multi drug resistant strains of microbes continue to increase at an alarming rate,[23] it is important to seek alternative antimicrobial agents from traditional medicinal plants to treat dental caries, periodontal disease and oral fungal infections.

The different parts of the *A. occidentale* have shown varying degrees of antimicrobial activity against pathogenic organisms. The potential uses of *A. occidentale* as an antibacterial agent against infections caused by *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Bacillus subtilis*, *Salmonella typhi*, *Escherichia coli* and *C. albicans* has been demonstrated. The methanol and aqueous leaf extracts and the methanol extract of the stem bark showed effective inhibition of these microbes supporting the traditional usage of *A. occidentale* in the treatment of various ailments.[24]

Alcoholic and aqueous extract of false fruit (cashew apple) of *A. occidentale* exhibited significant antimicrobial properties against gram positive *Bacillus cereus* (ATCC11778) and gram negative bacteria *Klebsiella pneumoniae* (ATCC11298).[25]

In the present study, petroleum ether, ethyl acetate and methanol extracts were effective against dental cariogenic pathogens namely *S. mutans*, *S. mitis* and *Lactobacillus species*. These extracts were ineffective against *S. salivarius*. The dichloromethane extract failed to demonstrate zone of inhibition against cariogenic pathogens and aqueous extract was effective only against *Lactobacillus species*. The cashew tree bark extract has shown antimicrobial activity against *S. mutans*, *S. mitis*, and *S. sanguis*, thereby suggesting its therapeutic use in dentistry as an antibacterial agent.[26] The ethanolic stem crude extracts of *A. occidentale* exhibited varying degrees of inhibitory effects against *S. mutans* associated with dental caries and the zones of inhibition increased with the increase in concentrations of the ethanolic stem crude extracts of *A. occidentale*, thus, exhibiting concentration dependent activity. The authors have suggested that susceptibility of *S. mutans* to ethanolic stem crude extracts of *A. occidentale* may be due to its Gram positive nature.[27] Thus it can be suggested that extracts of different parts of *A. occidentale* may be effective against cariogenic pathogens. Information on activity of *A. occidentale* against *Lactobacillus species* was not found in the literature.

In a study on antimicrobial effect of *A. occidentale* leaf extract on *Porphyromonas gingivalis* and *P. intermedia*, aqueous extract presented better antimicrobial activity than the menthanolic extract. In our study, methanolic extract was observed to be most effective against *P. intermedia* followed by petroleum ether exhibiting zone of inhibition 14mm and 10mm respectively. Similar results were observed in a study on leaf extracts of *A. occidentale* where methanolic extract was found to be highly active against selected pathogens like *Bacillus subtilis*, *Klebsiella pneumonia* and *E. coli* compared to aqueous extract.[24] The methanol extract of *A. occidentale* leaf was found to have higher inhibitory activity than ethyl acetate and petroleum ether and chloroform extracts were observed to be ineffective.[28] The highest activity of methanol extract of the leaves of *A. occidentale* against microbes may be due to the ability of methanol to extract a wider range of antibacterial principles than the aqueous solvent.[24]

All the 5 different extracts of leaves of *A. occidentale* did not exhibit antifungal activity against *C. albicans*. Similar results were observed with poor antifungal activity against *C. albicans* compared to Chlorhexidine.[29] On the contrary, methanol extract of leaves of *A. occidentale* was found to be effective against *C. albicans*.[24] In phytochemical screening of medicinal plants, the results may diverge as a result of the biochemical variations within species, geographical locations, methods or modes of extraction and solvent used.[17]

The phytochemical analysis of the stem bark of *A. occidentale* showed the presence of alkaloids and tannins.[24] The presence of tannins, alkaloids, saponins, terpenes and flavonoids[30] and also phenolics, steroid and sugar[31] in *A. occidentale* leaves has been demonstrated. Thus, the presence of the above phytochemical components may be responsible for the antimicrobial activity of the extracts on the test organisms.[24] Tannin is known to show the antibacterial activity by microbial proteins precipitation.[31] It has been suggested antimicrobial effect of tannins may be related to their ability to inactivate microbial adhesions and inhibit cell envelope transport proteins and hydrolytic enzymes such as proteases and carbohydrolases.[32] A few varieties of tannins had exhibited a strong antibacterial potential against *Porphyromonas gingivalis* and *P. intermedia* when the antimicriobial activity of six tannin components isolated from vaccinium vitis-idaea leaf extract were studied against selected periodontal pathogens like *P. gingivalis*, *P. intermedia* and *Actinobacillus actinomycetem comitans*.[33]

The present study has further proved the beneficial antimicrobial effects of leaf extract of *A. occidentale* against common pathogens present in the oral cavity. The further studies should be carried out on extracts of *A. occidentale* to purify and concentrate the active ingredient this medicinal plant to understand and explore the exact effect on pathogenic microorganisms.

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