ANTIFUNGAL ACTIVITY OF DMSO EXTRACTS OF TEN SELECTED HERBS USED FOR THE TREATMENT OF ORAL CAVITY INFECTIONS WITH REFERENCE TO ORAL CARCINOMA

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ABSTRACT

Introduction : *Candida albicans*, a diploid fungus also a casual agent of opportunistic oral infections in humans and is traditionally being treated using herbs. Due to increase in the antibiotic-resistant strains of microorganisms, traditional plants are being investigated for their antibacterial and medicinal values.

Aim: The aim of the present work was to explore the antimicrobial activities of selected plant leaves extracts against the pathogen *Candida albicans* which is also be responsible for causing oral cancer disease in recent studies. The aim of this study was to assess the antifungal activity of plant leaves extracts against *Candida albicans* and to study the inhibitory effect of chemical antifungal agents or drugs and elements of tooth pastes on *Candida albicans* pathogen isolated from oral cavity to compare with herbal plants extracts.

Material n methods: In present study, leaves extract of ten selected plant prepared in DMSO solution have been chosen for the investigation of in vitro antifungal activity which acts as expectorant and not having toxic properties on humans while for comparison or control, antifungal drugs have been taken.

Results: Results showed that *Candida albicans* shows most sensitivity towards the standard antibiotic cotrimoazol but very less towards other drugs like Fluconazole, minocycline, erythromycin respectively which indicated *Candida albicans* shows some resistance character towards drugs while the herbal extracts of *Lawsonia inermis*, *Withania somnifer*, *Curcuma longa*, *Cymbopogon citrates* and *Zingiber officinale* gives the best inhibitory effect and they have the potential to control growth of *Candida albicans*.

Conclusion: The present investigation was carried out to investigate the chemical and therapeutically potential by evaluating phytochemical and antifungal activity of the fresh leaves extracts. These findings will further help to develop the new antifungal drugs from these herbs or they can also be use in tooth pastes, oral ointments etc. for treatment of oral diseases at cheapest rate by inhibiting the growth of *Candida albicans*, an opportunistic fungal pathogen.

Key words: OSCC, *Candida albicans*, Medicinal Herbs, Antifungal Activity.

INTRODUCTION

An Oral Squamous Cell Carcinoma (OSCC) is the result of a multistage process of transformation of normal lesion to dysplastic lesions. A premalignant

or precancerous lesion is a morphologically altered tissue in which cancer is more likely to occur and includes oral leukoplakia, oral erythroplakia, and oral sub mucous fibrosis. Oral cancer being one of the most prevalent cancer, is a growing health problem around the world and is the second leading cause of the death. In India it is estimated that approximately 320,000 peoples die per year from cancers which gives the crude mortality rate of up to 38/100,000 and the prevalence of cancer at present is estimated to be 1.5 – 1.8 million. Oral cancer is now considered

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Figure 1 : Antimicrobial zone of inhibition (mm) against *Candida albicans* by leaves extracts of the selected herbs at different concentrations.

to be the most important contributor of increase in cancer morbidity and mortality rate. The magnitude of cancer in India warrants the close attention of researchers.

The *Candida* genus is a taxonomic grouping that was originally used to define, a ubiquitous fungi, are thin-walled, small (4 to 6 microns) reproduce by budding and are one of the most common causes of opportunistic mycoses worldwide. They can be recovered from environmental, human and other mammalian sources.

Candida sp., especially *Candida albicans*, an opportunistic pathogenic microorganisms which replaces the micro flora of oral cavity and its carriage

was also reported to be common in oral cancer patients^{1,2}. *C. albicans* is the most prevalent pathogenic species among all other clinically important *Candida* species like *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis* and *Candida dubliniensis* and is responsible for the majority of oral infections³. Candidal oral colonization (up to 93%) and infection (up to 30%) are frequently noted in oral cancer patients⁴. They have the potential to infect tissues within the body, however, these are most predominantly found on oral mucosa and have been suggested that *Candida albicans* species may be causative agents of oral cancer^{5,6}. Several potential virulence factors of *Candida albicans* species increases its pathogenicity like adhesion to

Figure 2 : Antimicrobial zone of inhibition (mm) against *Candida albicans* by positive and negative control (b).

host epithelial surfaces, secretion of proteinases enzyme and hyphal formation and penetration apparently the most significant.

To fight against fungal infections antibiotics are used as an important weapon which greatly benefited the health related quality of human life since their introduction but over the past few decades these health benefits are under threats as many commonly used antibiotics have now become less effective due to emergence of drug resistivity. *Candida* sp., also shows to exhibits resistance to antifungals which was firstly reported in 1995 and this has been reported by numerous other researchers also^{7,8,9}. Presently rise in candidal infections could also be due to a reflection of

inherently higher level of antifungal drug resistance in some *Candida* species. The exact mechanism of biofilm resistance to antifungals remains unclear, but it is probably multifactorial. The extracellular polysaccharide of the biofilm could serve as an inhibitor to diffusion of an antimicrobial agent or ionically bind the drug as it diffuses through the biofilm, thereby effectively reducing its bioavailability¹⁰. The infections caused by opportunistic fungi like *Candida albicans* are included under new spectrum of fungal pathogens. There is an increasing awareness amongst clinicians and microbiologists pertaining to importance of infection caused by opportunistic fungi (*Candida albicans*).

Table: 1 Antimicrobial inhibition zone mean (mm) by leaves extracts of the ten selected medicinal herbs at different concentrations against *Candida albicans*.

Leaf extracts	Volume of extract (ml)	100mg/ml	50mg/ml	25mg/ml	10mg/ml	5mg/ml
Lawsonia inermis	0.2	21 ± 0.13	19 ± 0.26	14 ± 0.17	12 ± 0.04	10 ± 0.22
Withania somnifera	0.2	20 ± 0.11	18 ± 0.08	15 ± 0.22	13 ± 0.34	12 ± 0.16
Zingiber officinale	0.2	24 ± 0.25	22 ± 0.15	18 ± 0.09	15 ± 0.43	13 ± 0.12
Curcuma longa	0.2	21 ± 0.57	18 ± 0.21	16 ± 0.42	13 ± 0.13	12 ± 0.06
Cymbopogon citrates	0.2	19 ± 0.49	18 ± 0.39	13 ± 0.19	12 ± 0.28	09 ± 0.21
Tamarindus indica	0.2	14 ± 0.09	13 ± 0.20	11 ± 0.18	09 ± 0.24	08 ± 0.36
Limonia acidissima	0.2	13 ± 0.26	13 ± 0.11	12 ± 0.31	10 ± 0.06	07 ± 0.04
Psidium guajana	0.2	14 ± 0.20	13 ± 0.18	12 ± 0.02	11 ± 0.17	10 ± 0.17
Annona reticulata	0.2	12 ± 0.19	12 ± 0.23	11 ± 0.34	09 ± 0.12	07 ± 0.17
Swertia chirata	0.2	11 ± 0.28	10 ± 0.18	10 ± 0.14	08 ± 0.29	07 ± 0.43

Table: 2 Antimicrobial inhibition zone (mm) by positive (drugs) and negative control (DMSO) of the against *Candida albicans*.

Drug / Control	Concentration of drug (µg/disc)/DMSO	Zone of inhibition (mm)	Resistance/ Sensitive
Fluconazole	10µg/disc	09	R
Cotrimoazol	25 µg/disc	18	S
Erythromycin	10 µg/disc	10	R
Minocycline	5 µg/disc	13	S
Negative Control- DMSO	0.2ml	-	R

Some natural compounds isolated from medicinal plants are a rich sources of novel anticancer drugs, have been of increasing interest since then. Some natural phenolic compounds play an important role in cancer prevention and treatment. Various bioactivities of phenolic compounds from medicinal herbs and dietary plants are responsible for their chemopreventive properties (e.g., antioxidant, anticarcinogenic, or antimutagenic and anti-inflammatory effects) and some inhibits the enzyme activities like histidine kinases responsible for candidal hyphae development¹¹. This situation forced to search for new antimicrobial substance from various sources including medicinal plants which now becomes essential to investigate newer drug with less resistance¹².

The aim of the work was to find the antimicrobial activities of plant leaves extracts prepared in DMSO against the opportunistic pathogen *Candida albicans* which is also be responsible for causing oral cancer disease in recent studies. The aim was to find some new components to develop the nontoxic antifungal drugs from these herbs or they can also be use in tooth pastes, oral ointments etc. for treatment of oral diseases at cheapest rate by inhibiting the growth of *Candida albicans*.

METHOD AND MATERIAL

Chemicals

Sabarouds Dextrose Agar (SDA), antifungal discs like Erythromycin (10µg/disc), Minocycline (5 µg/disc), Cotrimoazol (25µg/disc), Fluconazole (10µg/disc) were purchased from Himedia Pvt. Ltd., Mumbai, India. Other chemicals and reagents used for the study were of analytical grade.

Microorganisms

Micro-organism namely *Candida albicans* was collected from the Department of Oral Pathology

and Microbiology Laboratory, isolated from the oral cavity of oral cancer patients by sterile cotton swabs.

Plant material

Fresh and mature leaves of plants *Lawsonia inermis*, *Withania somnifer*, *Swertia chirata*, *Curcuma longa*, *Cymbopogon citrates*, *Tamarindus indica*, *Limonia acidissima*, *Psidium guajana*, *Annona reticulata* and stem of *Zingiber officinale* were collected from Department of Botany, University of Lucknow old campus, Lucknow during March- April 2015. Samples were collected in sterile plastic bag and brought to Mycology Laboratory of Botany Department, University of Lucknow, Lucknow, India.

Extract preparation

The leaves of plants were washed thoroughly with distilled water and dried at room temperature. Dried leaves were uniformly grounded using a mechanical grinder to yield fine powder. Ten grams of the powder was mixed with 100 ml of methanol in conical flask and kept in shaking incubator at 100 rpm for 24 hours. The mixture was filtered using Whatman filter paper no 1. Extract was dried and stored in an airtight container at 4°C until further use.

Preparation of extracts and isolated fractions for bioactivity test

Exactly 0.2g of crude DMSO (Dimethyl sulfoxide) extract was dissolved in 2ml DMSO (Dimethyl sulfoxide) to get 100mg/ml concentration. This was then serially diluted to obtain 50mg/ml, 25mg/ml, 10 mg/ml and 5mg/ml concentrations. This procedure was repeated for DMSO (Dimethyl sulfoxide) extracts using DMSO (Dimethyl sulfoxide) as a solvent for dilution. Similar dilution procedure was applied for the fractions corresponding to their yield. All of the fractions were prepared in two to three fold dilutions.

It should be clear that for those samples in which organic solvents were used for dilution, when antimicrobial test was done filter paper disks after impregnated in the stock solution was left to dry in flat glass (to let the solvent evaporated) and then it was sprayed with sterile distilled water.

Controls used in the study

Erythromycin (10µg/disc), Minocycline (5 µg/disc), Cotrimazole (25 µg/disc), Fluconazole (10µg/disc) were used as a reference or positive control for *C. albicans* and DMSO (Dimethyl sulfoxide) was used as a negative control for the study.

Antimicrobial activity assay

Agar disc diffusion method was performed for antifungal screening and broth dilution method for determination of minimum inhibitory concentration was applied as described elsewhere with little modification.

Agar disc diffusion method

Disc diffusion method was employed in the preliminary antimicrobial screening of both the crude organic extracts and bioassay guided isolated compounds. Test strains suspension of 0.5 McFarland was prepared from fresh cultures using normal saline. The plates were aseptically streaked with the test microorganism using a sterile swab and allowed to dry for few minutes. Sterile 6 mm diameter filter paper discs were impregnated with stock solutions (at concentrations 5, 10, 25, 50, to 100mg/ml). Using sterile forceps the discs were placed aseptically on the inoculated agar plates. The plates were then incubated for 24 hours at 37⁰ C. The experiments were carried out in triplicates. Presence of a clear circular zone around the sample impregnated disc was used as an indicator of activity. The results (mean values, n = 3) were recorded by measuring zones diameter in millimeters with the help of calipers. Disc impregnated with the solvent used DMSO was included as negative controls. For comparative purposes standard drug cotrimazole (25µg/disc) for antifungal was included as positive controls in the assays respectively^{13,14}.

Minimum inhibitory concentration (MIC)

The extracts, which showed superior antifungal activity in the agar disc diffusion method, were subjected to the MIC assay. The minimum inhibitory concentration (MIC) of the extracts was determined

for the test organisms in triplicates. To a 0.5 ml of varying concentrations of the extracts (20.0, 15.0, 10.0, 5.0 and 1.0mg/ml), 2ml of nutrient broth was added (so the extracts were dilute by a factor of 5). Therefore the final concentrations were 4, 3, 2, 1, 0.2, and 0 mg/ml as a control), and then a loopful of the test organism previously adjusted to 0.5 McFarland turbidity standard was introduced to the tubes^{15,16}. The procedure was repeated on the test organisms using the standard antibiotic (clotrimazole 40µg/disc). A tube containing nutrient broth only was seeded with the test organisms as described above to serve as control. Tubes containing cultures were then incubated at 37°C for 24h period. After incubation the tubes were then examined for candidal growth by observing the turbidity present in the tubes.

Data Collection

The antifungal activity of the selected ten herbs against fungus *Candida albicans* were obtained by measuring the diameters of the inhibition zones and compared them with that of the positive control drug Fluconazole, erythromycin, minocycline and cotrimazole and negative control DMSO. Antifungal activity was expressed as the mean zone of inhibition diameters (mm) produced by the herbs extracts.

RESULTS

Results showed that *Candida albicans* shows most sensitivity towards the standard antibiotic cotrimazole but very less towards other drugs like Fluconazole, minocycline, erythromycin (figure:2) respectively which indicated *Candida albicans* shows some resistance character towards drugs. However, the diameters of the inhibition zones by leaves extracts of DMSO solvents of selected herbs (*Lawsonia inermis*, *Withania somnifer*, *Curcuma longa*, *Cymbopogon citrates*, *Limonia acidissima*, *Tamarindus indica*, *Swertia chirata*, *Psidium guajana*, *Annona reticulate* and stem of *Zingiber officinale*) against the *Candida albicans* are different at different concentrations (Table:1, figure:1) among which the herbal extracts of *Lawsonia inermis*, *Withania somnifer*, *Curcuma longa*, *Cymbopogon citrates* and *Zingiber officinale* gives the best inhibitory effect and they have the potential to control growth of *Candida albicans* as they have diameter zone of inhibition above 12 mm at MIC 25mg/ml, 25mg/ml, 10mg/ml, 50mg/ml and 10mg/ml respectively.

Statistical Analysis

One way Analysis Of Variance (ANOVA) was used to determine the means, the standard deviations and the p-value. Student t- test was used to determine significance of the difference of the antifungal activities of the extracts. Results obtained in this study were expressed as mean inhibition zone (mm) $\pm$ S.D of three replicates. The mean and the S.D of each herbal extracts were used to compute the calculated t-value. Differences between the critical t-value and calculated t-values of the diameter of the inhibition zones of the herbal extracts on *Candida albicans* were computed. Among all the ten herbal species five (*Lawsonia inermis*, *Withania somnifer*, *Curcuma longa*, *Cymbopogon citrates* and *Zingiber officinale* gives) gives the alternate hypothesis because the calculated t-value was more than the critical t-value at p 0.05 hence there is a significant biological activity displayed by the compounds of these five herbs.

DISCUSSION

There are no drugs which can effect extremely to treat most oral cancers. Novel natural products offer opportunities for innovation in drug discovery. A considerable number of antitumor agents currently used in the clinic are of natural origin. In fact, natural products play a major role in cancer prevention and treatment by inhibiting the growth of responsible pathogen. For instance, over half of all anticancer prescription drugs approved internationally between the 1940s and 2006 were natural products or their derivatives¹⁷. Among them, plants have been the chief source of natural compounds used for medicine. The use of herbs and medicinal plant compounds to treat infections is an old age practice through out the world, especially in developing countries, where is there is dependence on traditional medicine for a variety of diseases. All the drugs from the plants are substances with the particular therapeutic actions extracted from the plants. The increased prevalence of antibiotic resistant fungal organisms is due to the extensive use of antibiotics may render the current antimicrobial agents insufficient to control some bacterial diseases and hence research for identifying novel substances that are active against human pathogens is an urgent need. The antimicrobial compounds produced by plants are active against plant and human pathogenic microorganisms. There is a general call for new emerging drugs that are highly effective towards cancer, possess low toxicity,

and have a minor environment impact. Many pharmacological investigations are also in race to identify new herbal drugs for the treatment of human diseases such as cancer and infectious diseases.

CONCLUSION

Presently there has been a significant increase in the incidence of human fungal infections especially candidal infections. Of the fungi regarded as human pathogens, members of the genus *Candida* are amongst the most frequently recovered from disease. The effective herbs can be used to treat infection at very low costs by using the effective herbal components in tooth pastes, oral gels etc. as by use of these products will totally vanishes the fungal colony from oral cavity without resulting in any side effects or toxicity like drugs. As use of chemical antimicrobial agents also enhances the resistivity character of microbes or fungus but these herbal products will not show any effect on enhancing resistivity character of this pathogenic fungus this could also be the best of its use. The findings of this suggest that these herbs (*Lawsonia inermis*, *Withania somnifer*, *Curcuma longa*, *Cymbopogon citrates* and *Zingiber officinale*) can be best used in treatment of oral infection by *Candida albicans* as they are natural, effective at MIC without any toxic effects. These herbs can be used in a form of oral gels, tooth pastes, oral ointments to vanish candidal infections or to inhibit its colonial growth at an early stage because they can proliferate in oral cavity, penetrate their hyphae in oral epithelium which may proves them to be very harmful.

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