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Research Article

ANTICARIOGENIC ACTIVITY AND HAEMOLYTIC STUDY OF SOME
MEDICINAL PLANTS LEAF PROTEIN EXTRACT AGAINST SIX ORAL
PATHOGENS IN *IN VITRO* CONDITION

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Abstract

Leaf extract of 12 medicinal plants were obtained by using 10mM sodium phosphate buffer and studied for their antibacterial activity against 6 cariogenic pathogens. Among them, the extract from *Streblus asper* showed the most intensive activity. Antibacterial activity of *Streblus asper* was investigated by the minimum inhibitory concentration (MIC) test and the viable cell count method. MIC of *Streblus asper* against *Candida albicans* causing dental caries was determined to be 2.5 µg/ml. The kinetic study showed that *Streblus asper* completely inactivated *C. albicans* at the concentration 50µg/ml in 15hours. Hemolytic study of the *Streblus asper* plant leaf protein gives HC₅₀ value at a very high range than that of the MIC values against *C. albicans* of the organisms and thus flourish its application as successful pharmaceutical drug in practice.

Keywords: Anticariogenic activity; *Streblus asper*; *Candida albicans*; haemolytic study.

Introduction

The human oral cavity is a habitat for about 500 cultivable and non-cultivable bacterial species (Paster *et al.*, 2001) Dental caries is a transmissible infectious disease (Tanzer, 1995). Although the human oral flora is quite diverse and complex, two species *Streptococcus mutans* and *Candida albicans*, have been implicated as the primary etiologic agents of dental caries (Loesche, 1986; Hamada and Slade, 1980).

The oral yeast tends to increase as a result of an increment in the number of retentive sites such as prostheses, orthodontic appliances and pacifiers (Ollila *et al.*, 1997) other factors such as the socioeconomic conditions seem to interfere in the colonization by *Candida* (Coulter *et al.*, 1993, Russel *et al.*, 1990). Persistent dental disease is painful, and most importantly, it has also been suggestively linked to diabetes, high blood pressure, heart disease, and multiple sclerosis later in life. The pain can be worsened by heat, cold, or sweet foods and drinks. Treatment often prevents further infection of the tooth structure. Early treatment is less painful than treatment of extensive decay.

Antibiotics such as penicillin and erythromycin have been reported to effectively prevent dental caries in animals and humans, but they are least used clinically because of resistance to these drugs by microorganisms. Recent natural remedies with the use of medicinal plants, which are good reservoirs of chemotherapeutants can be, contributed as an

alternative for antibiotic effects such as hypersensitivity reaction, supra infections, and teeth stainings.

Previous studies have demonstrated the antibacterial effect of plant extracts (Ross *et al.*, 1980; Deans and Richie, 1987; Mitscher *et al.*, 1987; Scheie, 1989; Heisey and Gorham, 1992; Shapiro *et al.*, 1994; Larsen *et al.*, 1996; Tichy and Novak, 1998; Van der Weijden *et al.*, 1998; Ishnava *et al.*, 2012) against oral bacteria. it has been well documented that medicinal plants confer antimicrobial activity towards oral bacteria. The literature survey of the folklore medicine reveals the use of stem of *Achyranthes aspera* for the treatment of tooth—ache and stem of *Mimusops elengi*, strengthens the gums. Despite several anticaries agents being available commercially, the search for an effective agent still continues. Natural products have shown to be a good alternative to synthetic chemical substances for caries prevention.

S. asper Lour is an herbal plant that belongs to the Moraceae family. This plant is found mainly in surrounding villages and open areas in the northern region of Gujarat. *S. asper* has been used in traditional medicine as decoction and pastes for wound infections. Several studies have reported that *S. asper* plant alcohol extract possess antibacterial and anti-inflammatory activities (Wongkham *et al.*, 2001) *S. asper* extracts have also been reported to possess anticancer activities

Though recent reports show the antimicrobial activity of *S.asper* against the cariogenic microbes, its antifungal

activity are screened in this study. Knowing the fact that little literature is available on the anticariogenic property of *S. asper*, the study is focused on assessing the plant extracts with different microbes. Once the antimicrobial property of the plant extracts is screened under *in vitro* condition against oral pathogens, *in vivo* trials can be carried out for the treatment of dental caries by external application on the caries tooth or as a preventive mouth rinse or chewing gum. Hence Anticariogenic Activity and Haemolytic Study of Some Medicinal Plants Leaf Protein Extract against Six Oral pathogens in *In vitro* condition was carried out.

Experimental Section

Plant material collection

Twelve Indian medicinal plants were selected for antimicrobial assay, based on their ethnomedicinal and traditional uses against infectious diseases based on literature survey and interaction with herbal healers. Leaves of *Azadirachta indica*(A), *Achyranthes aspera*(B), *Acacia nilotica*(C), *Aegle marmelos*(D), *Barleria prionitsi*(E), *Baliosperum montanum*(F), *Ficus benghalensis*(G), *Ficus religiosa*(H), *Ficus racemosa*(I), *Mimusops elengi*(J), *Streblus aspre*(K), *Solanum suratease*(L) were obtained from different places near New V.V. Nagar (Fig. 1).



Fig. 1: Leaves of various plants obtained near New V.V.Nagar. *Azadirachta indica* (A), *Achyranthes aspera* (B), *Acacia nilotica* (C), *Aegle marmelos* (D), *Barleria prionitsi* (E), *Baliosperum montanum* (F), *Ficus benghalensis* (G), *Ficus religiosa* (H), *Ficus racemosa* (I), *Mimusops elengi* (J), *Streblus aspre* (K), *Solanum suratease* (L).

Preparation of microbial suspension

Six pathogens *Lactobacillus acidophilus*, *Lactobacillus casei*, *Staphylococcus aureus*, *Streptococcus mutans*, *Candida albicans*, *Streptococcus pyogenes* were obtained commercially from the Microbial Type Culture Collection, INDIA. The stock of these microbes was kept in glycerol at -70 °C for further use. Growth media used in examining the samples at aerobic condition includes, nutrient agar (NA), Brain heart infusion, YEPD medium. Nutrient agar (NA) contained, per liter of deionised water. *L. acidophilus* was grown on medium containing Tomato juice: 100 mL, Yeast extract: 5.0gm, Skimmed milk: 100gm per 1000 mL of distilled water, pH- 7.2. *Lactobacillus casei* was grown on medium containing Peptone: 5.0gm, Yeast extract: 3.0gm, Beef extract: 3.0gm, dextrose: 1.0 gm, agar: 16.0gm per 1000mL of distilled water, pH-7.2. *Staphylococcus aureus*, *Streptococcus pyogenes* were grown on Nutrient agar containing Beef extract: 1.0 gm, Yeast extract: 2.0 gm, Peptone: 5.0 gm, NaCl: 5.0 gm, Agar: 15gm per 1000mL of distilled water, pH: 7.2-7.5. *Streptococcus mutans* was grown Brain Heart Infusion Agar containing Brain heart infusion: 5.0 gm, Tryptone: 10gm, Yeast extract: 10gm, Glucose: 10gm, Agar: 15gm per 1000mL of distilled water, pH- 7.2-7.5. *C. albicans* was grown on YEPD medium containing Yeast extract: 3gm, Pepton: 10 gm, Dextrose: 20 gm, Agar: 15 gm per 1000 mL.

Preparation of crude extract

All the plant leaves were thoroughly washed with tap water and shed-dried and used for extraction. Leaves were grinded in pre-cooled mortar and pestle with extraction buffer- 10mM sodium phosphate buffer in 1:3 ratios (shalaluddin *et al.*, 2011). This extract was centrifuged at 10,000 rpm at 4°C for 20 min. Then, extract was filtered. Supernatant was saturated with 80% ammonium sulphate. Now, this saturated crude extract was again centrifuged at 13,000rpm at 4°C for 30 min to pellet down the precipitated proteins. Pellet further in the buffer and subjected for purification of protein concentrate using dialysis bag. Dialysis bag was poured with protein to be purified and was put into the beaker containing 1 L of the extraction buffer and whole assembly was put on the magnetic stirrer for at least 3 hrs and then the buffer was changed. Finally all ammonium salts were removed out in the buffer, outside of the dialysis bag, and only protein suspended in the buffer remains inside the bag which can be directly subjected for further studies.

Antimicrobial Susceptibility Assay

Agar Well Diffusion Method

The antibacterial activity was studied by agar well diffusion method. Petri plates were prepared with 20mL of respective sterile nutrient medium for each organisms. A fresh bacterial culture of 100 µl having 10⁸ CFU/ml was spread on agar plates with sterile glass spreader. A well of 7 mm diameter punched off at previously marked petriplates in to agar medium with sterile cup borer and then it was filled

with 100 µl of respective plant leaves protein extract. Plates were placed for 30 minutes in refrigerator for diffusion of extracts and then incubated at 37 °C (or specified temperature) for 24 hours or more depending upon the organisms, until appearances of zone of inhibition. The zone of inhibition (excluding well diameter) was measured as a property of antibacterial activity. Antibiotics; ampicillin, amoxyillin and tetracycline, Ofloxacin were used as positive control at a concentration of 100 µg/mL.

Minimum Inhibitory Concentration.

The minimum inhibitory concentration (MIC) was performed according to the standard reference method. The extracts were dissolved in water +2% dimethyl sulfoxide (DMSO). The initial concentration of extract was 2.5 µg/mL to 100µg/mL. Each well was inoculated with suspension containing extract on 10⁸ CFU/mL of bacteria and fungi. The antibacterial agent penicillin and antifungal agent Amphotericin-B were included in the assays as positive controls. The plates with bacteria were incubated 24 h at 37°C. The MIC for bacteria was determined as the lowest concentration of the extracts inhibiting the visual growth of the test cultures on the agar plate. Three replications were maintained.

Kinetic study of crude protein extract

An overnight broth culture of pathogen was taken for the study and different assemblies were prepared for the kinetic study. Three different culture flasks were prepared. Blank – 50 ml of sterile fresh nutrient medium, Culture flask- 45 ml of fresh nutrient medium + 5 ml of pathogen, Test flask- 45 ml of fresh nutrient medium + 5ml of pathogen +3 ml of the crude leaf protein extract. All flasks were subjected to a shaker incubator at 37°C. The optical density (427 nm) was determined at every 60 minutes intervals for eighteen hours using spectrophotometer to obtain both the curves of normal growth in culture flask and inhibitory effect in the test flask for pathogen.

Haemolytic study of crude protein extract

5 ml of fresh human blood sample was taken using sterile disposable syringe and immediately washed with Phosphate Buffer Saline (PBS: 1.5mM KH₂PO₄, 2.7 mM KCL, 8.1 mM Na₂HPO₄, 135 mM NaCl, pH-7.4) and then centrifuged at 6000xg for 10 min. After washing four times with PBS (or until the supernatant was colourless), the human erythrocytes were re-suspended and diluted to 10 times of original volume with PBS, referred as stock erythrocyte suspension. Then, 1ml of stock erythrocyte suspension is incubated with 250µl, 500 µl, 750 µl, 1000 µl of *S. asper* crude leaf extract in four different vials for 60 min at 37°C. Two more vials were prepared as -ve and +ve control where stock human erythrocyte suspension will serve as -ve control and taken as blank with no haemolysis and stock human erythrocyte treated with equal volume of triton x-100 (1 v/v) will serve as +ve control with full haemolysis for the comparison of the effect of the protein extract. After the incubation period, the reaction mixtures were centrifuged at 1000 xg for 10 min to remove intact

erythrocytes. The 10 fold dilution of the supernatant of released haemoglobin was measured at 540 nm using spectrophotometer. The triplicate experiments were done. Hemolytic activity was expressed as a percentage haemolysis, which was calculated using the following equation:

$$\% \text{ Hemolysis} = \frac{A_{\text{sample}} - A_{\text{buffer}}}{A_{\text{max}} - A_{\text{buffer}}} \times 100$$

Where 'A_{Sample}' is A₅₄₀ of red blood cells with protein extract in stock human erythrocyte suspension, 'A_{Buffer}' is A₅₄₀ of red blood cells in PBS, and 'A_{max}' is A₅₄₀ of red blood cells with 1% (v/v) Triton X-100 in PBS. No haemolysis (0%) and full haemolysis (100%) were observed in the presence of PBS and 1% (v/v) Triton X-100, respectively.

Treatment with PROTEINASE K

In order to figure out whether antibacterial activity is the cause of proteins in the crude extract, the crude extract was treated with proteolytic enzyme; Proteinase K (Wang et al., 2006). Proteinase K was added into 200 µl of crude extract in which the protein amount ratio of protein substrate and proteolytic enzymes equaled to 1:8. The treatment reaction was performed at 37°C for 20 hr. After treatment, the supernatant was obtained by centrifugation at 10,000 rpm for 5 min and used for antibacterial activity assay.

Result and Discussion

In the present study, The result of sensitivity of crude leaf protein extracts against the tooth decaying organisms (CA, LA, LC, SA, SMU and SP) were assessed by visualizing the presence or absence of inhibition zone and measuring diameter of zone of inhibition. The antifungal activity of the above-mentioned plant extracts was tested against *C. Albicans*. Out of 12 plants leaf protein extracts; 10 plant leaf

protein extracts has shown the activity against *C.albicans*. Maximum activity was against *Streblus asper* (36mm). and minimum activity against *Mimusops elengi* (19mm) Inhibitory effect of *Streblus asper* is same as of antibiotics .Out of all the 12 plant leaf protein extracts, except *Ficus racemosa* leaf protein extract- (17mm) no other plant has shown activity against *Lactobacillus acidophilus*. All the 12 plant leaf protein extracts found to be inactive against *Lactobacillus casei* .Only *Mimusops elengi* (9mm) was active against *Staphylococcus aureus*. *Achyranthes aspera*, *Aegle marmelos*, *Ficus benghalensis* *Mimusops elengi* and *Solanum suraltease* (9mm) shown activity against *staphyococcus mutans*. Only leaf protein extract *Solanum suraltease* (9mm) inhibited *Streptococcus pyogenes*. The results are summarized in Fig. 2.

Although use of micro-dilution in determining anti-Candida activity is the best way (Makimura et al., 1998,) other methods such as agar diffusion and paper disc diffusion could be utilized as a screening method or in combination with a micro-dilution assay (Kawamura et al., 2004). In this study the anti-Candida activities were assessed by measuring the diameter of the zone around the agar wells; in other studies discs with different diameters were used (Hammer et al., 1998). The smaller or bigger zone around the colony is related to the sensitivity or resistance of the fungi to the tested materials. Because there are differences in measurement of the transparent zone and also because different amounts and dilutions of the tested materials were used, comparisons are difficult. MIC was determined as the lowest concentration of the extract, which inhibited the growth of the tested microorganisms. Results exhibit the profound and promising activity of *S.asper* against *C.albicans*. Fungus on YEPD medium showed MIC at 2.5µl.

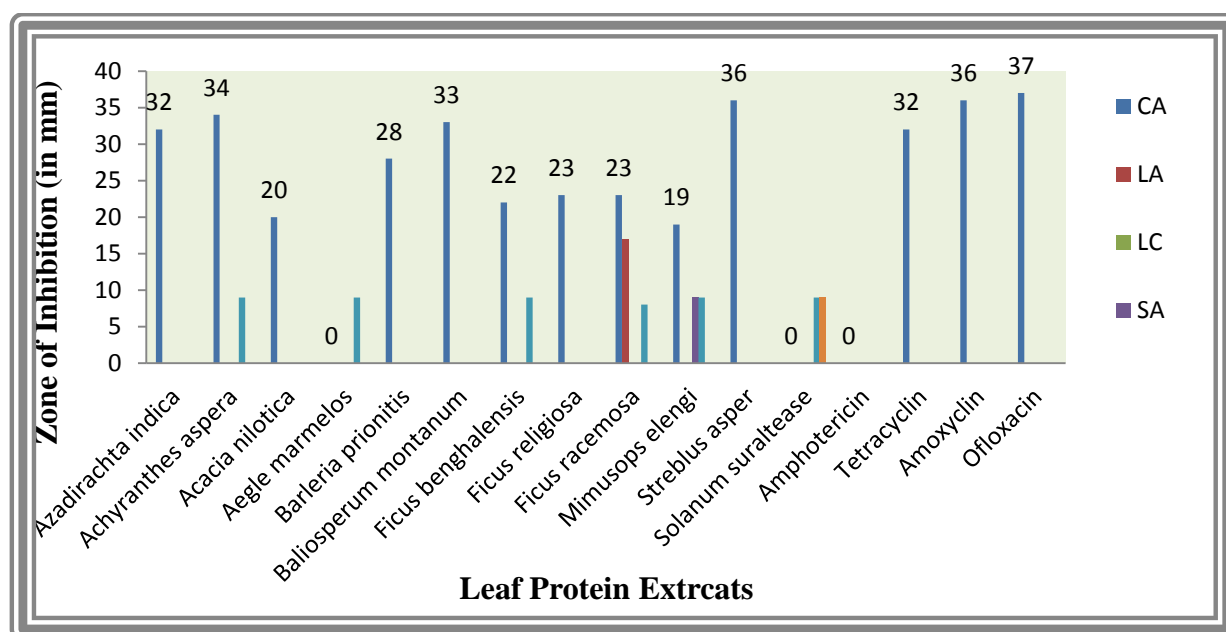


Fig. 2: Antibacterial activity of leaf protein extracts against cariogenic microorganisms (Zone in mm).

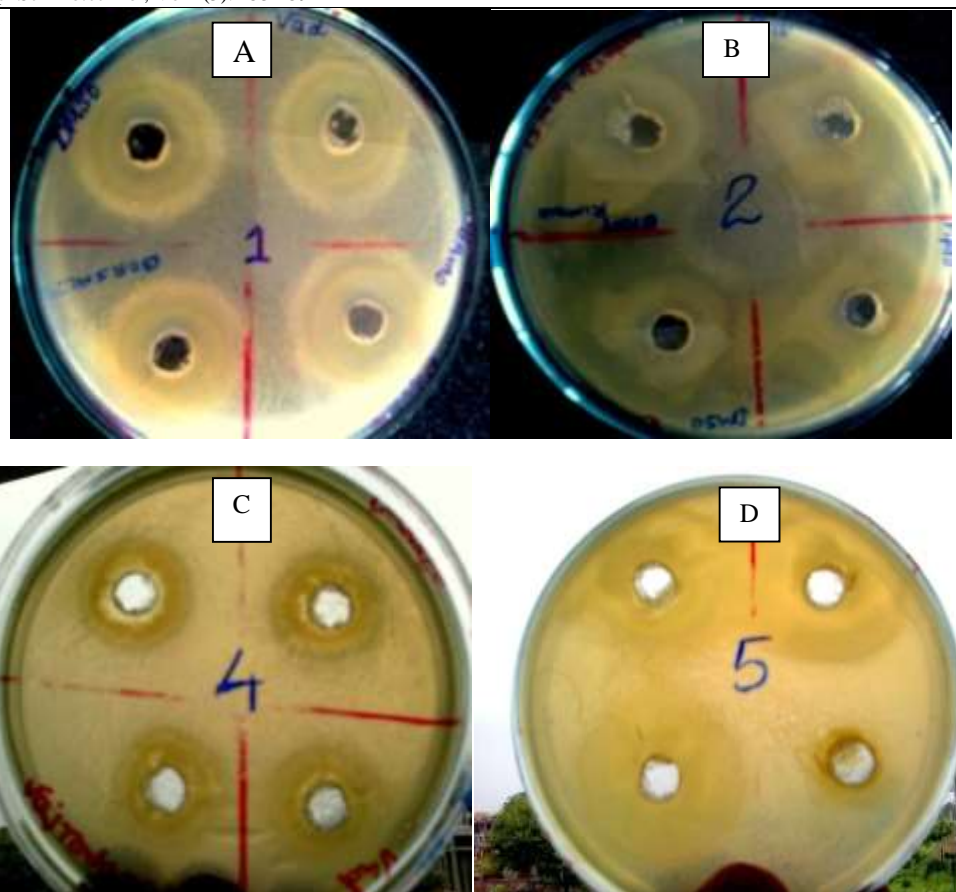


Fig. 3: Antimicrobial activity of *Candida albicans*

- A. Inhibition zone for *Candida albicans* of *Ficus benghalensis*, *Acacia nilotica*, *Mimosops elengi*
- B. Inhibition zone for *Candida albicans* of *Solanum surattease*, *Aegle marmelos*, *Ficus religiosa*
- C. Inhibition zone for *Candida albicans* of *Ficus racemosa*, *Barleria prionitis*
- D. Inhibition zone for *Candida albicans* of *Streblus asper*

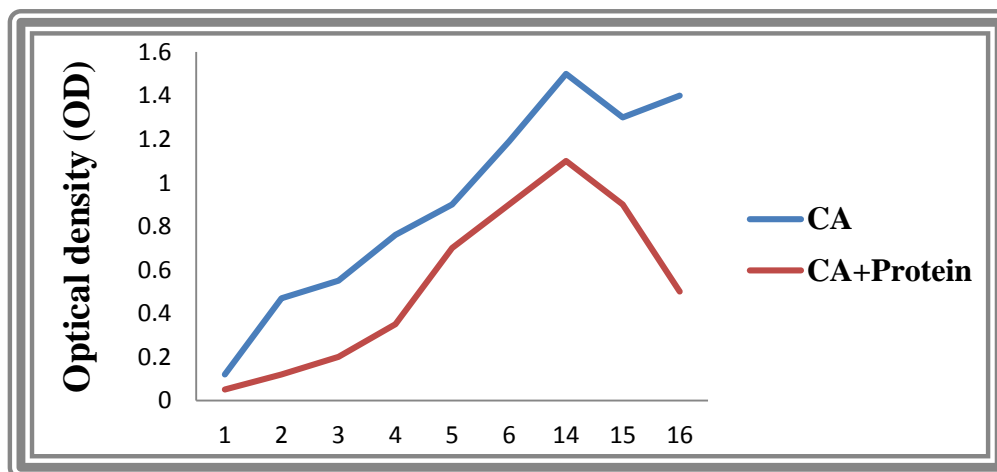


Fig. 3. Kinetics of antimicrobial activities of leaf protein extract of *Streblus asper* against *Candida albicans*

The organism *C.albicans* when exposed to the plant leaf extracts at doses equivalent to MIC; demonstrates decline in population after 15 hours of exposure.(figure 3)The extract might be formulated in a delivery vehicle such as chewing-gum, gel or vanish that can sustain its release over a period of time

In clinical terms, it could be of interest to evaluate the relationship between the virulence and the degree of

hemolysin production among pathogenic and commensal isolates of *Candida* using this method. Haemolytic activities of the *Streblus asper* plant leaf protein gives HC₅₀ value at a very high range than that of the MIC values against *Candida albicans* of the organisms and thus flourish its application as successful pharmaceutical drug in practice (Fig.-4)

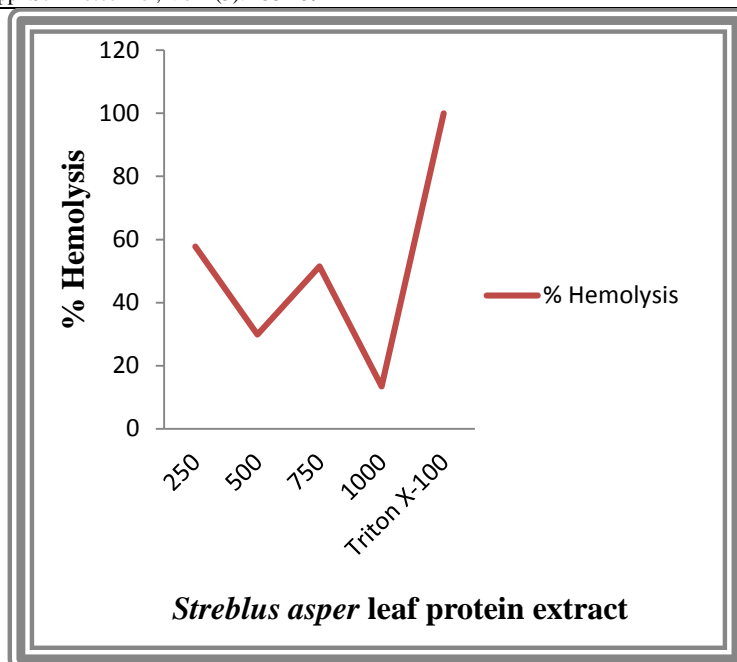


Fig. 4: The susceptibility of freshly collected erythrocytes to hemolysis (%) with leaf protein extract of *Streblus asper*

The *Streblus asper* crude leaf protein extract after treatment with protein hydrolytic enzymes, Proteinase K revealed no further activity of the extract against *Candida albicans* which conforms the antimicrobial activity was only in the presence of fully structured protein and after its destruction or hydrolysis by enzyme; the activity vanish.

Table 1: Treatment with Proteinase K

Samples	Loaded protein (µl)	Inhibition zone (mm)
Crude extract	100	36
Crude extract + Proteinase K	100	0

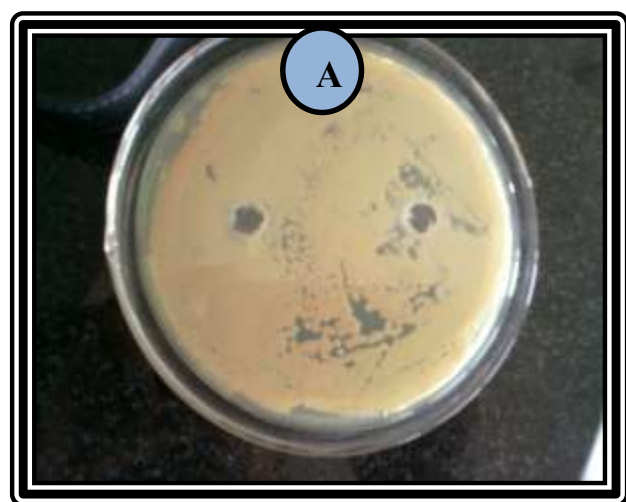


Fig. 6: Treatment with Proteinase K

- A. Absence of inhibition zone for *Candida albicans* of *Streblus asper* when treated with Proteinase K

Conclusion

From the experiment we could conclude that *Streblus asper* has high inhibitory action against *Candida albicans* than

other extracts. Due to resistance against present antibiotics by microbes, we need to compare antifungal drugs with plant-derived ones, which are cheaper, safer and more nature-friendly (Lopez *et al.*, 2001). Although the anti-*Candida* activities of essential plant extracts are different due to factors such as environmental conditions, extraction methods and non-standardized processing, we will hope for the futures of these products (Chaieb *et al.*, 2001)

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