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RESEARCH ARTICLE

ISOLATION OF *ACTINOBACILLUS ACTINOMYCETUMCOMITANS* (AGGREGOBACTER) AND *PORPHYROMONAS GINGIVALIS* FROM SUDANESE AGGRESSIVE PERIODONTITIS PATIENTS AND TESTING INHIBITORY EFFECT OF GARLIC EXTRACT COMPARED TO CHLORHEXIDINE DIGLUCONATE 0.2%.

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Abstract

Aggressive periodontitis is a destructive inflammatory condition affecting the investing and supporting tissues of the teeth. Detectable levels of *Porphyromonas gingivalis*, *T. forsythia*, *Prevotella intermedia*, *Campylobacter* and *Actinobacillus actinomycetumcomitans* are associated with disease progression. Adjunct use of antimicrobial agents was implemented for the treatment of the disease and also some antiseptic mouth wash like chlorhexidine digluconate 0.2%. Now a day's medicinal plants are used as antimicrobial against bacteria due to increase of bacterial resistant to antibiotics, so Garlic (*Allium sativum*) used as antimicrobial, antifungal and antiviral against oral pathogens. So the aim of the present study was to isolate *Actinobacillus actinomycetumcomitans* and *Porphyromonas gingivalis* from Subgingival fluid of Sudanese aggressive periodontitis patients and to study the effect of water garlic extract against the isolate compared to chlorhexidine digluconate 0.2%.

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Introduction:-

Thirty aggressive periodontitis patients were recruited from patients attending Khartoum Teaching Dental Hospital after written approval. Patients gave verbal consent for clinical examination, age range 18-35 years. Patients examined for plaque index (PLI), Gingival Index (GI), Probable pocket depth and attachment loss in mm.

Microbiologically: Sub-gingival plaque samples were obtained from two deepest sites per quadrant for each patient for identification of the major causative micro-organisms *Actinobacillus actinomycetumcomitans*, and *Porphyromonas gingivalis*. Identification was done by culture. Garlic extract was subjected to preliminary antimicrobial screening using the cup-plate diffusion method against the detected bacteria. The Minimum inhibitory concentration (MIC) of the garlic extract was determined, Chlorhexidine digluconate 0.2% used as positive control and distilled water as negative control (invitro study). Zones of inhibition were measured and averaged. The mean diameter of the zone of inhibition in mm was compared to the zone of inhibition of chlorhexidine using Pearson correlation test to identify if there is a statically difference between garlic extract and chlorhexidine digluconate 0.2%. Correlation is significant at the 0.01 and 0.05 level (2 tailed)

Results: There was an increase in plaque index, gingival index, pocket depth and loss of attachment among aggressive periodontitis patients. *Actinobacillus actinomycetumcomitans* was not detected (negative) but

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Porphyromonas gingivalis detected in 70% of the sample. There was growth inhibition of *Porphyromonas gingivalis* by water garlic extract compared to chlorhexidine digluconate 0.2%. There was no statistical significance difference between the means. **Correlation is significant at the 0.01 level (2 tailed). Pearson correlation of Garlic and chlorhexidine digluconate = 0.596 and P-Value = 0.069.

Conclusion:-

The present study indicates that garlic extract has the same effect of chlorhexidine digluconate 0.2% against *Porphyromonas gingivalis*, so it may have therapeutic value in prevention and treatment of aggressive periodontitis. So garlic extract can be used in well designed clinical trial in form of mouth wash for prevention and treatment of aggressive periodontitis.

Key words: Garlic; Chlorhexidine digluconate 0.2%; Oral bacteria; *Porphyromonas gingivalis*.

Introduction:-

Aggressive periodontitis is a destructive inflammatory condition affecting the investing and supporting tissues of the teeth. It is classified into localized and generalized form. (1) Detectable levels of *Porphyromonas gingivalis*, *T. forsythia*, *Prevotella intermedia*, *Campylobacter* and *Actinobacillus* *Actinomycetemcomitans* are associated with disease progression and their elimination by therapy is associated with an improvement in the clinical signs of the disease. (2,3) Both *Porphyromonas gingivalis* and *Actinobacillus* *Actinomycetemcomitans* are known to invade host tissue cells which may be significant in more aggressive forms of adult periodontitis. (4) *A. actinomycetemcomitans* are gram-negative bacilli which are small to coccoid on solid media. Bipolar staining on Gram stain may occur. Growth in culture is slow and requires 2 – 7 days, the organism grows on enriched media such as blood or chocolate agar, and prefers 5-10% CO₂ atmosphere. Selective media have been described, but are not used routinely. Colonies of *Actinobacillus* *Actinomycetemcomitans* are small at 24 hours, with an opaque dot at the center of the colony, which will develop into a star-like shape on prolonged incubation. Important characteristics that lead to proper identification include the Gram stain morphology, slow growth, negative oxidase and positive catalase results, negative spot indole results, and the ability to ferment glucose but not lactose. The oxidase test of the isolate reported as weakly positive by some laboratories, a characteristic which is present in up to 19% of strains, and makes the distinction from closely related species, particularly *Pasteurella* and catalase-positive *Haemophilus* species difficult. *A. actinomycetemcomitans* is closely related to *H. aphrophilus* and *H. paraphrophilus*, but previous proposals to transfer this organism to the *Haemophilus* genus were rejected. (5,6) *Porphyromonas gingivalis* belongs to the genus *porphyromonas* from family bacteroidaceae, these bacteria are dark-pigmented gram-negative strict anaerobic coccobacilli, they grow in culture media forming convex, smooth glossy colonies of 1-2 mm diameter, which demonstrate a progressive darkening in the center, because of production of protoheme, the substance responsible for the typical color of these colonies. For identification culture plated on Brucella (Oxoid) 5% Sheep Blood Agar with Vitamin K and Hemin, plates were cultivated in an anaerobic atmosphere at 37°C for 48h and to obtain pure culture black pigmented colonies were recultivated at the same conditions and then a biochemical test was performed. (White and Myrand 1981) (7) the major virulence factors of *porphyromonas gingivalis* is Lys-gingipain, a lysine-specific cysteine proteinase, which interferes with both cell-matrix and cell-cell adhesion, fimbriae of *Porphyromonas gingivalis* play an important role in its adhesion to and invasion of host cells and has an effect on periodontal ligament cells through a cooperative action of gingipains. (8, 9, 10) There are many trials to use vaccine against *porphyromonas gingivalis* to aid in prevention of periodontitis in animals but it is still in trials. (11,12) The treatment of aggressive periodontitis in addition to plaque control and debridement, adjunctive treatment of aggressive periodontitis includes the use of systemic antibiotics e.g. tetracycline oral capsules or by tetracycline fiber therapy, minocycline and metronidazole (13,14,15,16). Some antiseptic mouth wash used after scaling and root planning and after surgical treatment, like Octenidine and Chlorhexidine digluconate 0.2% which is used as mouthwash or gel instead of toothpaste. Chlorhexidine digluconate has many disadvantages of staining the teeth, it has a powerful taste, suppresses normal sense of taste in few people, irritant to the oral tissues and may produce sore lips (17,18). Also ozonated water used as antimicrobials in the treatment of periodontitis patients (19). With increase of bacterial resistance to antibiotic medicinal plants is applied widely for the treatment of many diseases, one of these plants is garlic which was used as an antimicrobial agent due to the presence of allicin (sulphur compound) called thiosulphonate, the plant uses allicin as an antimicrobial against invaded bacteria or single cell pathogen. (20,21). Garlic used in different forms, powder, oil and heated garlic which has an antimicrobial effect on *Staphylococcus aureus* B33. Garlic compounds were used as, antiviral, antifungal and antiprotozoal, it has antibacterial effect on *Escherichia*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Klebsiella*, *Proteus* and *Mycobacterium*. (22,23,24) Allicin showed synergistic effect when combined with antibiotics which may overcome the resistance of micro-organisms. (25) In dentistry, garlic used as antimicrobial against oral bacteria in

the form of mouth wash which have inhibitory effect against Gram-positive, Gram-negative organisms and fungi by reducing bacterial counts. (26) In some studies tea tree oil, garlic, might be an alternative to chlorhexidine solutions against oral microorganisms. (27,28) Filter sterilized, aqueous extract of garlic has the ability to inhibit the growth of gram positive and gram negative micro-organisms and it has action against the arg and lys-gingipains activity of *P. gingivalis* and it has activity against multidrug-resistant (MDR) strains of *Streptococcus mutans* isolated from human carious teeth and inhibit *P. gingivalis* protease activity. ((29,30,31,32) .Garlic extracts in concentrations of 12.5% is cytotoxic to human gingival fibroblasts but it is safe at 6.25, 3.12, and 1.5% , the high concentration of garlic is less cytotoxic than chlorhexidinedigluconate. (33) In Sudan there are many studies on the prevalence of aggressive periodontitis among different age groups, the *A. actinomycetemcomitans* were detected in the subgingival plaque of 12 (70.6%) patients with aggressive periodontitis and from only one (5.9%) control subject, showing a significantly higher frequency of detection in cases than in controls. The JP2 clone of *A. actinomycetemcomitans* was not detected in the subgingival plaque of high school subjects in Sudan. The detection of non-JP2 types of *A. actinomycetemcomitans* may be a useful marker of increased risk for development of aggressive periodontitis in young subjects. (34)

so the present study was designed to investigate the periodontal health of the aggressive periodontitis patients including determination of major causative micro-organisms, *Actinobacillus actinomycetemcomitans* and *porphyromonas gingivalis* and to test the effect of a garlic extract on their inhibition compared to chlorhexidine 0.2 % (in invitro study).

Material and method:-

Cross-sectional analytical clinical and microbiological hospital based study at Department of Periodontology Faculty of Dentistry, University of Khartoum. Thirty aggressive periodontitis patients both males and females Patients were within the age group 18-35 years (mean 25.4±5.5 years) newly diagnosed according to clinical and radiographic examination as aggressive periodontitis patients For all participants the clinical examination was done using a mouth mirror and periodontal probe (University of Michigan-0 probe) for 10 teeth recorded in special form .The patients were examined for plaque accumulation according to PLI (Sillness and Loe)(,35) examination of gingivitis according to GI (Loe and Sillness)(36), in addition to measuring pocket depth and loss of attachment in mm for the deepest site of the examined teeth. Panoramic X-rays or periapical were done for all patients. Patients excluded if they had systemic diseases, those who were immunocompromised, pregnant ladies, patients used antibiotics 3 months before the examination and patients who had periodontal treatment 3 months before the start of the study.

Ethical approval:-

Ethical clearance was given by the Research Committee (Faculty of Medicine/ University of Al-Neelain 2007) and approval from the director of faculty of dentistry , head department of periodontology for sample collection . Since the patient are adult, seeking periodontal treatment and capable of giving informed consent, voluntary and freely they agrees verbally to participate in the study after explanation of the aim of the study .

Bacterial isolate:-

Isolation of *Actinobacillus actinomycetemcomitans*:

Bacterial suspension was vortex and homogenized and incubated anaerobically with cooked meat media. To obtain pure culture Samples were subcultured in blood agar with vancomycin 5Mg/ml and incubated anaerobically in anaerobic jar at 37C° using gas generating kits for carbon dioxide (Micromaster Laboratories PVT. LTD38/39, Kalpataru Ind Estate, Thane (W) MAH. INDIA.

Isolation of *porphyromonas gingivalis*:

Bacteria incubated anaerobically in blood agar plates for 48 hours and 7-14 days in anaerobic jar at 37C using gas generating kits for carbon dioxide (Micromaster laboratories PVT. LTD 38/39, Kalpataru Ind Estate, Thane(W) MAH.INDIA .To obtain pure culture the isolate was sub-cultured in Kanamycin blood agar (by dissolving 1.5 g Kanamycin in 100ml of sterile water and then mixed with blood agar.

Bacterial identification:-

Colony feature:-

Characterization of bacteria was done based on colony features, size, color, shape and consistency.

Gram stain:-

Bacterial film fixed and stained with Triphenylmethane dye, Crystal violet, in conjunction with iodine solution and subsequently treated with an organic solvent as alcohol and counter stained with safranin. Bacteria which retain the dye are designated Gram positive and the gram negative bacteria lose the dye.

Simplified routine biochemical tests for identification of bacterial isolates:-

Biochemical tests will be done under anaerobic condition (in anaerobic jar) The biochemical activities of the purified isolates were then studied for identification and confirmation of these organisms. The biochemical tests carried out include Fermentation tests:(Harris,1964).(37) Methyl red tests,Voges-Proskauer test,Indole production test: Hydrogen sulphide production test). Catalase test, Oxidase test:(Cruickshank et al ., 1975, Salle,1961.(38,39) Urease test (Cheesbrough, 1996).(40) Biochemical tests for *P.gingivalis*, important characteristics leading to proper identification include , negative oxidase and positive catalase results, negative spot indole results, and the ability to ferment glucose but not lactose.

Culture characteristics:-

It grows as white, translucent smooth non-hemolytic colonies in blood agar colonies sticky and adherent M not ferment g/lucose, catalase, hemolysis and urease positive ,oxidase,indole and vogesproskauer negative.

Isolation of *prophryomonasegingivalis*:-

Bacteria incubated anaerobically in blood agar plates for 48 hours and 7-14 days in anaerobic jar at 37°C using gas generating kits for carbon dioxide (Micromaster laboratories PVT. LTD 38/39, KalpataruIndEstate,Thane(W)MAH.INDIA .To obtain pure culture the isolate was sub-cultured in Kanamycin blood agar (by dissolving 1.5 g Kanamycin in 100ml of sterile water and then mixed with blood agar.

Microscopical examination showed gram -ve rods .

Biochemical tests:

None of the isolates fermented glucose, All isolates were urease positive, vogesproskaur negative. All isolates were indole -ve, catalase positive and oxidase negative. Some of isolates make gelatin hydrolysis.

Results:-**Result of Isolation of *Actinobacillusactinomycetumocomitans*:**

Actinobacillusactinomycetumocomitans not detected in the samples (Negative result)

Result of Isolation of *prophryomonasegingivalis*

Microscopical examination: Gram negative, short rod

Culture characteristics On blood agar, black hemolytic colonies were seen after 48 hours incubation under anaerobic condition at 37°C. Sample sub-cultured in blood agar with Kanamycin for 7-14 days anaerobically, colonies changed to brown.

Biochemical tests:-

Most isolates ferment glucose do not ferment sucrose and lactose,oxidase negative, catalase positive, β hemolytic and indole negative

Water garlic extract:-

Fresh garlic cloves (100g) were blended in 20 ml distilled water filtered using cotton wool and ultrafiltered under reduced pressure by using a Buchner funnel and a side armed flask. By subtracting the weight of insoluble material from the weight of the original cloves, the final concentration of garlic extract in solution was determined to be 16.5% (w/v). The garlic extract was stored at -20°C and used for antibacterial testing.

In vitro testing of extract for antimicrobial activity:-**Determination of minimum inhibitory concentration (MIC):-**

The antibacterial activity of *Allium sativum* was evaluated by cup-plate agar diffusion method for aqueous extract. The garlic extract used in present study was 16.5% (w/v). One ml of the standard bacteria suspension were thoroughly mixed with 60 ml of molten sterile Muller Hinton agar media and poured into the petridishes. The agar was left to set and in each of plate cups (10mm diameter) were cut using a sterile cork borer and agar discs were removed. Garlic extract were prepared in series of increasing concentrations. The bottom of each plate was marked off into 8 segments. Serial dilution from 100%, 50%, 25%, 12.5% to 10% is used. 50 μ l of extracted garlic

introduced into the wells using automatic microlitre pipette and all plates were incubated at 37°C for 24 hours. Chlorhexidine 0.2 % (Claradine ,Mfd by MEDPHARMA,United Arab Emirates) used as a positive control, the negative control were distilled water .Sensitivity of *P.gingivalis* and control was determined by measuring the diameter of the zone of inhibition. The end point (MIC) is the least concentration of antimicrobial agent that completely inhibits the bacterial growth. The resultant growth inhibition zones were measured averaged and the mean values were tabulated. (Table 1).

Table (1) Screening for antibacterial activity of garlic against the clinical isolates *Prophyromonasgingivalis* Compared to Chlorhexidine (Mean diameter of of inhibition zone)

water garlic extract Concentration%	Mean Diameter of zone of inhibition (mm)	negative control (distilled water)
10.0 %	13.6± 0.51	—
12.5 %	14.2± 0.63	—
25.0 %	15.7 ±1.4	—
50.0 %	18.4±1.26	—
100.0 %	21.5±1.77	—
Chlorhexidine Digluconate	0.2 %	25±2.2

Values were expressed as mean ± S.D.

(-) No growth

The data were carried out using a computer software program (SPSS version 16, Chicago, USA). ANOVA tests were used to identify significant differences between the means of the study groups. Critical *P* values of significance were set at 0.05 and a confidence of 95%. Pearson Correlation and the value expressed as mean± S.D .*P* value at 0.5

Result:-

The extract inhibited the growth of *P.Gingivalis* at concentration of 100%,50%,25%, 12.5% and 10%. The end point(MIC) which is the least concentration of garlic that completely inhibits the growth of *P.gingivalis* was 10%. The sensitivity pattern as zone of inhibition (mm) of aqueous garlic extract concentration showed growth inhibition activities against *Prophyromonasgingivalis* compared to sensitivity pattern in (mm) of positive control chlorhexidine 0.2%. The pearson correlation between water garlic extract and chlorhexidine 0.2% is 0.596 and , *p* value > 0.069,so There was no significance difference between aqueous garlic extract and chlorhexidine 0.2%. No zone of inhibition around the negative controls, (distilled water).Table(2)

Table 2

	Garlic water extract	Chlorhexidine
Garlic water extract Pearson correlation	1	.596
Sig.(2-tailed) <i>P</i> value		.069
N	60	10
Chlorhexidine Pearson correlation	.596	1
Sig.(2-tailed) <i>P</i> value	.069	
N	10	10

*Correlation is significant at the 0.05 level (2 tailed)

**Correlation is significant at the 0.01 level (2 tailed)

Pearson correlation of Garlic and Chlorhexidine = 0.596
P-Value = 0.069

Discussion:-

In the present study *Actinobacillus actinomycetumcomitans* was not detected so it is in agreement with previous reports which did not support the association of *actinobacillus actinomycetumcomitans* with aggressive periodontitis; Mohammad Hossein et al (2004), in which no periodontopathogenic bacterial growth was observed in 14 of the samples (6.2%), Faverir M. et al (2007), José Roberto Cortelli (a)2010 and to some extent with Lau I (2004) in which they found that *Actinobacillus actinomycetumcomitans* was the least recovered micro-organisms from samples taken from patients with aggressive periodontitis. In the present study *p.gingivalis* was detected in 70% of

aggressive periodontitis patients and the result is almost in agreement with the previous studies of Savit E.D. et al (1988) in which the detection was 91% , Ann L ,et al (1998) in which *P.gingivalis* was detected in 79% of the patients and in 25% of healthy subjects . The authors of those studies concluded that *P.gingivalis* is implicated in the pathogenesis of periodontitis and aggressive periodontitis but *P.gingivalis* may not be a normal inhabitant of periodontally healthy subjects .The results are also in agreement with those of Atsuo Amano (1999) in which *P.gingivalis* was detected in examined sample of 73 patients (78.5%) with aggressive periodontitis ,as well as the results of the study by Lau L, et al (2004) in which *P.gingivalis* was low in healthy subjects and increased in patients with periodontitis . For the prevention and treatment of periodontal diseases a number of topically applied antimicrobial agents were found to inhibit the formation of bacterial plaque and hence , prevents the onset of gingivitis and periodontitis. Chlorhexidine gluconate was commonly used in 0.2% mouthwash or gel. With the rise of bacterial resistance to antibiotics, there is need to search for other antimicrobial agents for treatment and control of oral infections. Garlic which is known to have antibacterial antiviral, anti-protozoal and antifungal activity (Tariq Abdullah 1987). It has an inhibitory effect against gram+ve and gram-ve bacteria in oral cavity (Elnima et al 1983), (Grosso 2002) inhibition of *streptococcus mutans* (Bakri 2005) in study of the inhibitory effect on putative periodontal pathogens and (Fanni et al 2007) in study of inhibition of multidrug-resistant strain of *streptococcus mutans*, so in the present study , which is the first study in Sudan, we confirmed the marked growth inhibition activity of garlic (grows in northern Sudan) against *P. gingivalis* which has been described before (Bakri 2005). Aqueous extract (concentration 16.5%) at serial dilution of 100%, 50%, 25%, 12.5% and 10% showed activity . there was no significant difference between Chlorhexidine gluconate 0.2%, and the aqueous extract , The MIC of the aqueous extracts of garlic in the present study was 10% .

Conclusion:-

Since garlic water extract in the present study had invitro inhibitory effect on *P.gingivalis* isolated from the examined group of aggressive periodontitis patients compared to chlorhexidine and due to the widespread use of antibiotic and the spread of antibiotic resistance ,we present evidence for the antimicrobial activity of garlic extract against *P.gingivalis* and this raises the possibility that garlic may have therapeutic use for prevention and treatment of the onset of aggressive periodontitis.

Recommendation:-

- 1-Further laboratory studies on the effectiveness of garlic extract on periodontal pathogens are needed.
- 2-Preparation of Garlic in dosage forms (mouth wash or local ointment) to be tested on volunteers patients with aggressive periodontitis in carefully designed clinical trials.

Limitation of study:-

Since allicin is the main biologically active component part of garlic extract and garlic extract showed inhibition for growth of *P.gingivalis*, so the extracts should be subjected to GC-MS analysis or High Performance Thin Layer Chromatography to determine the amount of allicin .

Competing interests:-

The authors declare that they have no competing interest.

Financial competing interest.

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Authors contribution:-

Professor Ghandour contributed in conception and study design and supervising the study.

Professor Al Nima contributed in laboratory work.

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