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Effectiveness of *Azadirachta indica* bark extract in periodontitis induced rabbit model

Gul-e-Nazish, Muhammad Shahzad, Liaqat Ali and Noman Johnson

Abstract
Background and Aim: *Azadirachta indica* twigs have been used to maintain healthy periodontium for many years in Pakistan and India. Leaves and fruit oil have also been used to treat and as a prophylactic agent for periodontitis in the form toothpastes, mouthwashes, oral chips. However, there is lack of scientific data for its efficacy in this particular condition. The present study was designed to see the effectiveness of Azadirachta indica bark extract in rabbit model of experimentally induced periodontitis.
Experimental procedures: 24 rabbits were distributed into four groups. Group A served as healthy control, Group B, C and D were induced with periodontitis. Group C and D were treated with Azadirachta indica bark extract and amoxicillin, respectively. Parameters recorded were periodontal index, total leukocyte count in blood and gingival crevicular fluid, erythrocyte sedimentation rate and levels of IL- 6 in serum and gingival crevicular fluid.
Result: The treatment with Azadirachta indica bark extract and amoxicillin significantly reduced inflammation. There was a significant reduction in bleeding on probing, plaque accumulation, probing pocket depth, total leukocyte count in gingival crevicular fluid and blood; also levels of IL 6 in gingival crevicular fluid were reduced in both treatment groups.
Conclusion: Results indicate that Neem (*Azadirachta indica*) bark extract possesses the capacity to improve periodontal inflammation.

Keywords: *Azadiracta indica*, periodontitis, amoxicillin, *Porphyromonas gingivalis*, interleukin 6

1. Introduction
Incidence of chronic periodontal inflammation varies with age between 50 to 60 years. An overall prevalence of 87.4% has been reported in Pakistan during year 2008 [1]. Pathogenesis of periodontitis includes two separate but interrelated components: One is pathogenic bacteria and other is host response component. Body's own cells respond to bacterial products by producing inflammatory mediators such as tumor necrosis factor-α and interleukins (IL-1, IL-6, and IL-8). These mediators cause destruction in healthy periodontal tissue [2, 3]. One of the first events to occur during bacterially induced periodontal inflammation is accumulation of neutrophils in soft periodontal tissues. Hydrolytic enzymes released from these neutrophils then cause irreversible damage to the gingiva, alveolar bone, cementum and dentin, eventually leading to tooth loss [4]. Routine scaling, root planning, antibiotics and NSAIDs are current standard treatments for periodontitis. However, these routine treatments are highly inconvenient and painful techniques with least patient compliance. Commercially available antibiotics (penicillin and cephalosporin, erythromycin, tetracycline and derivatives and metronidazole) show increased resistance by pathogenic bacteria. Risks of opportunistic infections in immuno-compromised patients also exist. These antibiotics can alter oral microbial composition and have undesirable side-effects such as vomiting, diarrhea and tooth staining. Hence, the exploration for alternative treatment products with significant safety index continues. Phytochemicals from medicinal plants are isolated and used as good alternatives to synthetic products [5]. *Azadirachta indica* (neem) is a common, evergreen tree; belongs to the family *Meliaceae*. The importance of *Azadirachta indica* has also been acknowledged in a report entitled "Neem a tree for solving global problems", published in 1992 by U.S. National Academy of Science Approximately 135 compounds have been isolated from different parts of the *Azadirachta indica* tree. These compounds have been classified as isoprenoid and non-isoprenoid [6]. Each component of the *Azadirachta indica* tree exhibits some medicinal property. Significant advancement relating biological activity and medicinal uses of *Azadirachta indica* has been achieved. *Azadirachta indica* tree is now taken as valuable source for the development of unique natural medicinal and industrial products. It has become a cynosure of modern medicine also it has been used in Ayurveda, unani and homoeopathic medicine on a large scale [7].

Azadirachta indica has been utilized as preferred aid for maintaining healthy periodontal tissue for thousands of years in India and South Asia. Leaves have been extensively used for treating gingivitis and periodontitis. The most probable anti-inflammatory mechanism of *Azadirachta indica* is by inhibiting prostaglandin E and 5HT (serotonin receptors). Mechanical plaque-removing efficacy of *Azadirachta indica* chewing sticks is close to that of a standard toothbrush. It is reported that the miswak possess antibacterial and plaque growth inhibiting properties by the release of a variety of beneficial chemicals such as fluoride, saponins and sterol [8]. Hence, this study was designed to see the relationship between pathophysiology of periodontitis and effectiveness of *Azadirachta indica* bark extract.

2. Materials and Methods

The study was accomplished at Pharmacology Laboratory, University of Health Sciences, Lahore. Animal care and experimental procedures were done at institutional experimental research laboratory. The research was conducted in accordance with the internationally accepted principles for laboratory animal use and cares as found in for example the European Community guidelines (EEC Directive of 1986; 86/609/EEC) or the US guidelines (NIH publication #85-23, revised in 1985). 24 male New Zealand rabbits approximately 1-year-old and weighing between 1 to 1.5 kg, were divided into four groups by balloting method. Group A was control healthy group. Group B was induced with periodontitis. Group C was induced with periodontitis and treated with *Azadirachta indica* bark extract while Group D was periodontitis induced and treated with amoxicillin. All animals were fed with normal rabbit feed and water ad libitum. They were kept in the animal house under standard conditions of temperature (22-24 ºC), humidity (45-65%) and natural day and night cycle. The experimental techniques were followed according to institutional animal care guidelines.

2.1 Materials

1. Bark of *Azadirachta indica*
2. Amoxicillin

2.2 Preparation of *Azadirachta indica* Bark Extract

Azadirachta indica bark was collected from garden of Railways Walton academy, Lahore and after identification by plant taxonomist; reference specimen was carried to Pharmacology Laboratory University of health sciences, Lahore. The external hard wood was removed. Remaining material was washed, air dried and cut into small pieces. Then dry pieces were milled to form fine powder. 1 kilogram of *Azadirachta indica* bark powder was soaked in 4 liters of distilled water for twenty-four hours at room temperature with periodic shaking. The aqueous extract was filtered through Whatman qualitative Grade 1 filter paper. Thick brown extract containing approximately 70g of total powder was obtained when aqueous extract was concentrated in water bath. The extract was stored at 4 ºC in a dark brown bottle to avoid biological degradation [9].

2.3 Induction of periodontitis

Experimental periodontitis was induced by placement of ligatures along with topical application of *P. gingivalis* isolated in laboratory [10]. Ten patients with chronic periodontitis were selected from the outpatient department, Section of Periodontology, Punjab dental hospital, Lahore. Eighteen sub gingival plaque and gingival crevicular fluid samples were collected from periodontal patients with the help of micropipette and transport swabs. The samples were inoculated into 2ml of Brucella broth (Acumedia, USA) augmented with 0.4-microlitre/milliliter vitamin K (Sigma-Aldrich, USA) and 5-microgram/milliliter hemin (Sigma-Aldrich, USA). They were diluted and plated onto Trypticase soy agar (Sigma-Aldrich, USA), augmented with 10% defibrinated sheep blood, 5-microgram/milliliter hemin (Sigma-Aldrich, USA) and 0.4-microgram/milliliter vitamin K (Sigma-Aldrich, USA). The plates were incubated for three days at 37 ºC in jars with anaerobic environment supported through palladium-catalyzed hydrogen-carbon dioxide envelopes (Gas Pak Plus; BD Microbiology Systems, USA). The bacterial colonies were isolated according to size, color, and shape and staining. The anaerobic bacteria were recognized by the API 20 and rapid ID (bio Merieux, France). Long wave UV light was used to distinguish between *P. gingivalis* and other black-pigmented, anaerobic, gram-negative rods. Absence of fluorescence was supposed to be a rapid taxonomic test to identify *P. gingivalis*. Isolated colonies were cultured in anaerobic environment whole night at 37 °C in Schaedler's broth (Acumedia, USA) augmented with vitamin K 0.4-microlitre/milliliters (Sigma-Aldrich) and 5-microgram/milliliter hemin (Sigma-Aldrich, USA). Spectrophotometer was used to decide bacterial number at 600 nm, adjusted to 109 CFU (optical density 600 nm) [11].

Ligatures were placed around mandibular incisors using 3-0 silk suture under general anesthesia with 40mg/kg ketamine (Global pharma, Pakistan) IV and 5mg/kg b. w. Xylazine (Sigma-Aldrich, USA) injections (IM). *P. gingivalis* (109 CFU) was mixed with carboxymethylcellulose (Sigma-Aldrich, USA) and this slurry was applied topically to the suture ligated mandibular incisors of Group B, C and D three times a week for total 6-weeks. Periodontitis was diagnosed using "periodontal index". The parameters recorded were probing gingival pocket depth (PPD) between 4 mm to 6 mm (PPD 4-6 mm), presence or absence of calculus/plaque, bleeding on probing [12].

2.4 Treatment Protocol

A. indica bark extract 500mg /kg body weight dissolved in 2.5ml distilled water was given for seven days three times a day via oral route using 5 cc disposable syringe to animals of group C. Amoxicillin 20mg/kg body weight dissolved in 2.5ml of distilled water, was given for seven days, three times a day via oral route using 5 cc disposable syringe to animals of group D.

2.5 Sample collection and technique

Blood samples of all groups were obtained from the marginal auricular vein on seventh day after treatment with *A. indica* bark extract and amoxicillin respectively in serum separating tubes with help of 5 cc syringe. Gingival crevice fluid was collected from deepest pockets (5mm). The periodontitis induced and treated teeth were separated with cotton rolls and dried with air. Supragingival plaque was removed. The periopaper absorbent strips were infixed into the crevice until mild resistance was felt 0.5 to 30 seconds were given to gingival crevicular fluid to absorb. Then strips were removed from crevice. Samples stained with blood were discarded. Gingival crevicular fluid volume was determined using a Periotron 8000. The strips were immediately placed in eppendorf tubes containing 150µl of phosphate elution buffer with composition (0.05% Tween 20, NaCl 8 gram/liter, KCl

0.2 gram/liter, Na2HPO4 1.44 gram/liter, KH2PO4 0.24 gram/liter) (Merck, USA). Samples were transported to the laboratory, stored at −80 °C until analysis. All samples were eluted by vortexing for 30 min followed by centrifugation at 10,000 RPM for 10 minutes when analyzed [13].

2.6 Recording of clinical parameters
It was gross analysis of periodontal tissue with the help of "periodontal index" using "Michigan" periodontal probe in all groups (A, B, C, D) on last day of treatment and 30 days post treatment. Parameters recorded were bleeding, absence or presence of calculus/Plaque and depth of pathological pocket (Queiroz *et al*., 2008). Qualitative variables (Bleeding and Plaque/calculus) were assigned numbers such a Nil = 0, Mild = 1, Moderate = 2 and Severe = 3.

2.7 Inflammatory cells in blood and crevicular fluid
Total leukocyte number was counted in 1ml of the blood sample and 100µl of the GCF sample already diluted with phosphate buffer solution with the help of hemocytometer through 10x objective of microscope [14].

2.8 Interleukin 6 Levels
The IL-6 was analyzed in the serum and GCF samples of all groups by ELISA (Koma- Biotech, Korea).

3. Statistical analysis
The results were analyzed by using statistical package for social sciences (SPSS) version 16. One-way ANOVA was applied to observe the difference in groups. Post hoc Tukey test was used to see which groups mean differs from the other. Mean ± SD was given for normally distributed quantitative variables. A p-value ≤ 0.05 was taken as statistically significant.

4. Results
4.1 Treatment with *Azadirachta indica* showed improvement in clinical parameters
4.1.1 Effect of *Azadirachta indica* bark extract on bleeding
A significant increase in bleeding on probing in group B was seen ($p < 0.001$). Treatment with *Azadirachta indica* bark extract ($p < 0.001$) and amoxicillin ($p < 0.05$) significantly ameliorated the bleeding as compared to group B (Fig. 1).

4.1.2 Effects of *Azadirachta indica* bark extract on calculus/plaque deposition
Plaque accumulation in group B was increased ($p < 0.001$) as compared to control group A. Treatment with *Azadirachta indica* bark extract ($p< 0.001$) and amoxicillin ($p < 0.05$) significantly lowered the presence of plaque/calculus (Fig. 2).

4.1.3 Effect of *Azadirahcta indica* bark extract on gingival pocket depth/mm
Probing pocket depth was increased in group B ($p < 0.001$) as compared to control group. Treatment with *Azadirachta indica* bark extract ($p < 0.001$) and amoxicillin ($p <0.001$) significantly reduced probing pocket depth as compared to diseased group (Fig. 3).

4.2 Effects of *Azadirachta indica* on inflammatory cells in GCF
A significant increase in total leukocyte count in group B ($p < 0.001$) was seen as compared to control group A. Treatment with *A. indica* bark extract ($p < 0.001$) and amoxicillin ($p < 0.001$) significantly reduced leukocyte count in GCF sample as compared to diseased group B (Fig. 4).

4.3 Effects of *Azadirachta indica* bark extract on inflammatory cells in blood
Group B showed an increased count of inflammatory cells ($p< 0.001$) as compared to control group A. Leukocyte number was significantly reduced in *Azadirachta indica* bark extract treated group C ($p <0.001$) and amoxicillin ($p < 0.001$) treated group D as compared to group B (Fig. 5).

4.4 Effects of *Azadirachta indica* bark extract on IL 6 (pg/ml) level in GCF (n=6)
Levels of IL 6 were increased in group B ($p< 0.001$) when compared to group A in gingival crevicular sample. Treatment with *Azadirachta indica* bark extract ($p < 0.001$) and amoxicillin ($p< 0.001$) reduced IL 6 levels equally in group C and D after treatment when compared with group B (Fig 6).

4.5 Effect of *Azadirachta indica* bark extract on serum IL6 level
No significant change was found in the serum level of IL 6 in all groups A, B, C and D (Fig. 7).

4.6 Effects of *Azadirchta indica* on Erythrocyte Sedimentation Rate
ESR was increased in group B ($p < 0.001$) from normal value of group A. Values were equally decreased in groups C ($p < 0.001$) and D ($p< 0.001$) after treatment with *Azadirachta indica* bark extract and amoxicillin, respectively (Fig 8).

5. Discussion
Azadirachta indica has been extensively used for periodontitis treatment and found to be effective in reducing dental plaque and gingivitis [15]. Current study was designed to observe the efficacy of *A. indica* bark extract in treating periodontal disease. Periodontal index was used to observe clinical parameters in the form of bleeding, calculus/plaque deposition and clinical attachment levels of gingiva to the alveolar bone. The bleeding on probing (BOP) is a broadly used clinical sign of the periodontitis and disease development. Absence of BOP is considered as a reliable indicator of periodontal stability [16]. In this study, we observed a significant reduction of bleeding on probing with *A. indica* treatment. Gingival tissue became firm in texture, pink in colour and no bleeding was observed as compared to diseased group where gingival tissue was swollen, red and easily bleeding on minute touching with probe. Amoxicillin treated group showed significant reduction in bleeding from diseased group but lesser than that of *A. indica* treated group. *A. indica* group showed better results on calculus/plaque accumulation as compared to amoxicillin treated group. According to our data *A. indica* bark extract proved itself as a plaque inhibitory agent. It prevents adhesion of biofilm to hydroxyapatite crystals on tooth enamel surface. This fact has also been previously documented by [17]. Probing pocket depth is one of the important parameters for determining periodontal healing. *A. indica* treated group during post-treatment follow up showed improved clinical reattachment as compared to amoxicillin treated group. Evaluation of all clinical parameters between different groups provides a precedence of *A. indica* bark extract over traditional therapy. Our results of clinical parameters are comparable to the study conducted by [18].

Total leukocyte count is an important parameter to check progression of periodontal pathology. *A. indica* bark extract significantly lowered total leukocyte count in gingival crevicular fluid and serum. Previously [19] have reported that *A. indica* seed oil possesses anti-inflammatory property similar to NSAIDs such as indomethacin. Elevated IL-6 levels in GCF of diseased group were observed while its levels were reduced equally by *A. indica* extract and amoxicillin. Our results confirmed a correlation between IL-6 levels and pathogenesis of periodontitis [20]. No difference was observed among serum IL-6 levels of all four groups. This may be due to high expressions of IL-6 mRNA in gingival mononuclear cells (localized) but not in peripheral mononuclear cells [21]. However, more investigations with IL-6 mRNA expression from inflamed gingival tissue and from GCF sample at different sampling time are required. Erythrocyte sedimentation rate is a nonspecific measurement of inflammation. Our findings showed a slight increase in ESR in diseased group which went down to normal in both treatment groups. Amoxicillin is known to be effective against a large variety of infections caused by many Gram positive and gram negative micro-organisms in both human and animals [22]. Amoxicillin has an indirect role in termination of inflammation by removing pathogenic bacteria. Our findings with amoxicillin (reduction in bleeding, ESR and leukocyte count) were due to its antibacterial action against *Porphyromonas gingivalis* [23].

Table 1: Clinical parameters

Parameters	Group A	Group B	Group C	Group D
Bleeding	0.3±0.5	2.6±0.4^{a}	0.16 ±0.4^{b}	1.16±0.7^{b}
Calculus/plaque deposition	0±0	2.6±0.5^{a}	0.5±0.5^{b}	1.4±0.5^{b}
Pocket depth/mm	0.8±0.4	5±1.2^{a}	1.5±0.6^{b}	4.1±0.6

Clinical parameters (Mean±SD) recorded with the help of Periodontal index in all groups (n=6), Where a shows a significant difference with control group A and b shows a significant difference with periodontitis induced group B

Table 2: Total leukocyte count

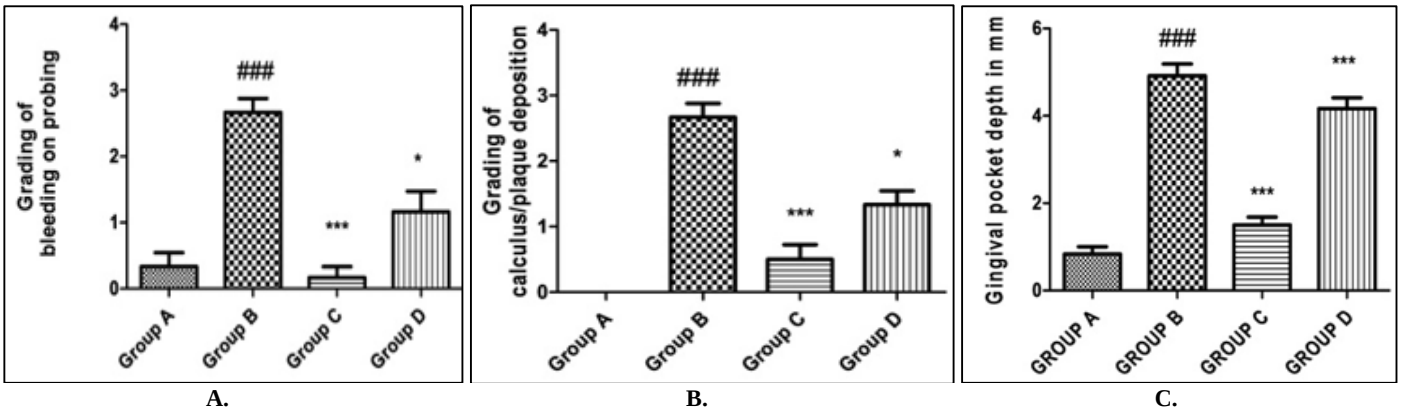
TLC/mm^3	Group A	GroupB	Group C	Group D
GCF	10±28.2	1025±233.9^{a}	14±4.9^{b}	168±80.10^{b}
Blood	7.6±1.21 ×10^{3b}	14.8±2.2 ×10^{3a}	4.3±0.51×10^{3}	4.1±0.41×10^{3b}

Table shows (Mean±SD) of inflammatory cells in all groups (n=6), Where a shows a significant difference with control group A and b shows a significant difference with periodontitis induced group B

Table 3: IL 6 and ESR

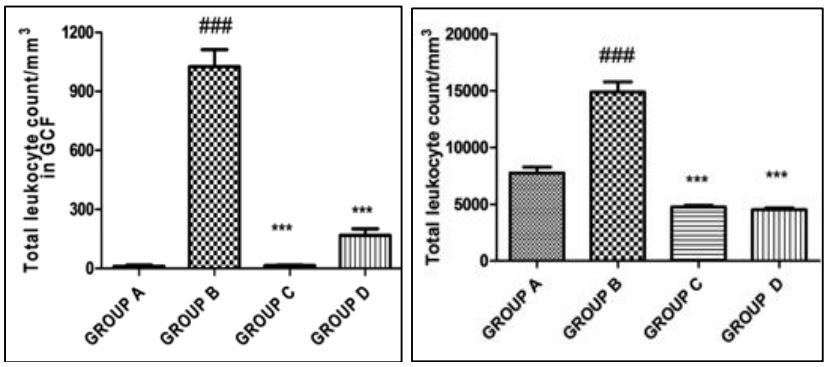
IL-6 (pg/ml)	Group A	Group B	Group C	Group D
GCF	0.033±0.001	0.089±0.007^{a}	0.033±0.003^{b}	0.031±0.002 ^{b}
Serum	0.033±0.002	0.033±0.005	0.032±0.001	0.033±0.005
ESR mm/hr	4.6±0.81	7.6±1.5^{a}	5±0^{b}	5±0^{b}

Table shows (Mean ± SD) of IL-6 level in all four groups (n=6), Where a shows a significant difference with control group A and b shows a significant difference with periodontitis induced group B.



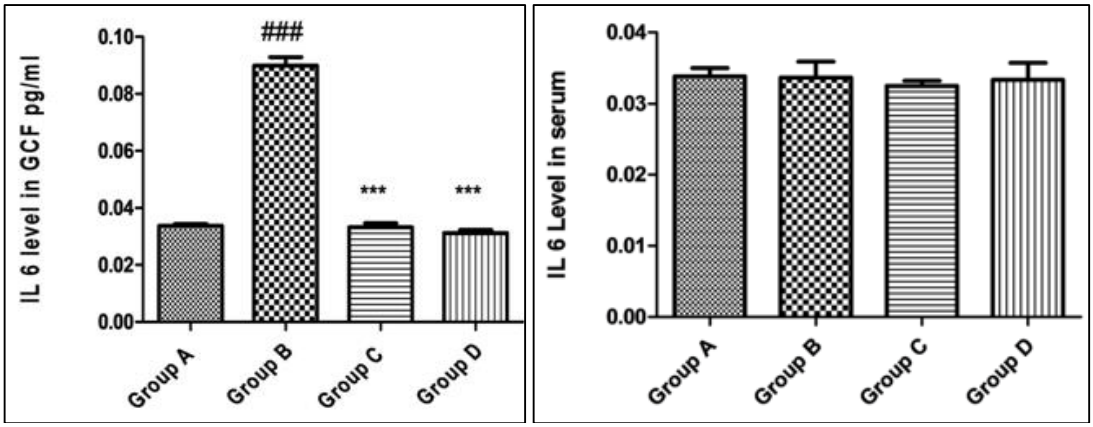
A. **B.** **C.**

Fig 1: Clinical parameters: Graph A presents bleeding on probing in all groups (n=6). Significant reduction in bleeding with *Azadirachta indica* treated group was seen. While graph B presents calculus/plaque deposition in all groups (n=6).Calculus/plaque deposition was less with *Azadirachta indica* treated group. Graph C presents probing pocket depth in all groups (n=6) again significant improvement was seen with *Azadirachta indica* treated group



A. **B.**

Fig 2: Total leukocyte count: Graph A presents total leukocyte count in GCF in all groups (n=6).Significant reduction in cell number was seen with *Azadirachta indica* treated group. Graph B presents with total leukocyte count in blood in all groups (n=6). TLC was raised with periodontitis induced group and decreased down with both *Azadirachta indica* and Amoxicillin treated groups.



A. **B.**

Fig 3 IL 6: Graph A presents IL 6 levels in all groups (n= 6).IL 6 levels were increased with periodontitis induced group and decreased down with *Azadirachta indica* and Amoxicillin treated groups. Graph B presents IL 6 levels in all groups (n=6).NO significant difference was seen in the levels of IL 6 in all four groups.

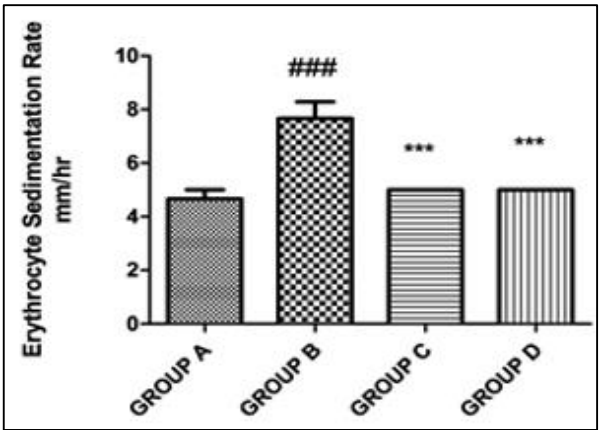
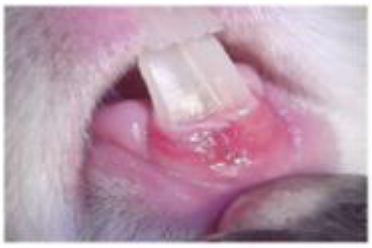


Fig 4: Erythrocyte Sedimentation Rate: Graphical presentation of ESR values in all four groups (n= 6). ESR values were decreased with both *Azadirachta indica* and Amoxicillin treated group.

A. **B.** **C.** **D.**



E.

Fig 5: Photographs: Healthy gingival tissue, firmly attached to teeth surface **A** (control group n= 6). Redness, swelling, bleeding, presence of plaque and pathological Pocket **B** (periodontitis induced group n= 6). Healing of gingival tissue, reduction in gingival pocket depth **C** (*Azadirachta indica* treated group n= 6).Post treatment follow up showing pink gingival tissue, reduction of plaque accumulation on tooth surface, fine reattachment of connective tissue **D**. *(Azadirachta indica* treated group n= 6).Presence of moderate redness and swelling of gingival tissue, presence of pathological gingival pocket **E**. (Amoxicillin treated group n=6)

6. Conclusion

We concluded that aqueous extract of *Azadirachta indica* bark extract and amoxicillin at doses of 500mg/kg b.w. and 20mg/kg b.w. respectively, reduced gingival bleeding, swelling and total leukocyte count in GCF and serum samples. Moreover, *A. indica* extract and amoxicillin also reduced levels of IL-6 in GCF samples of periodontal lesions. In addition, *A. indica* bark extract also showed a potent anti-plaque activity. Antimicrobial activity of *A. indica* bark extract against *Porphyromonas gingivalis* was comparable to amoxicillin. However, further studies should be carried out to see the effectiveness of *A. indica* bark extract especially on antimicrobial and immunomodulatory aspects. Pharmacokinetic properties of *A. indica* should also be taken under consideration.

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