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Antimicrobial effect of flaxseed (*Linum usitatissimum*) on periodontal pathogens: An *in vitro* study

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Abstract
Usages of antibiotics play an adjunctive role in the management of periodontitis. Antibiotic resistance is a major global issue, implicated with inadvertent drug usage. Herbal interventions are a therapeutic strategy that warrants greater research attention. Flax seed is one such recognized original super food which is rich in omega 3 fatty acids and has demonstrated potent anti-microbial and anti-biofilm activity. This *in vitro* study aims to evaluate antimicrobial activity of flaxseed extract against periodontal pathogens. Ethanolic extract of flaxseed powder was prepared and the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans* and *Tannerella forsythia* were estimated. Flaxseed extract exhibited bacteriostatic activity against all pathogens whereas bactericidal against *P. gingivalis* at the concentration of 100µl/ml. Present study demonstrated bactericidal role of flaxseed against *P. gingivalis*, a key periodontal pathogen can be an adjunct to periodontal therapy.

Keywords: Flax seeds, Periodontal pathogens, Periodontitis, Omega-3-fatty acids

1. Introduction
Periodontitis is the core dispute that may act as nidus of infection and inflammation for the causation of various other diseases. According to WHO Global Oral Health Data Bank, approximately 10-15% of adults are affected with periodontitis worldwide [1]. Non-surgical periodontal intervention may require antibiotic coverage, but more often it is associated with antibiotic resistance crisis which is one of the tremendous threats to global health [2]. Resistance to antibiotics has been reported with the emergence of resistant pathogens that endangers the beneficiary action of antibiotics [3]. Hence, herbs with medicinal properties are effective source of treatment in various diseases and are unlikely to develop resistance as compared to that of antibiotics. The usage of natural extracts continues to amplify all over the world for treatment of various health challenges since Vedic periods. One such ancient wonder food is Flaxseed which had played a role in Ayurvedic medicine for thousands of years. In the 8th century, King Charlemagne, a Holy Roman emperor believed the health benefits of flaxseed so strongly that he passed law that required his subjects to consume it regularly [4] Flaxseed (*Linum usitatissimum*), commonly known as flax or linseed, a member of the genus *Linum* in the family Linaceae, which is one of the richest source of omega-3- polyunsaturated fatty acids (ω-3 PUFA), moisture, fat, energy, fibers, proteins, carbohydrates, vitamins and minerals. It is also eminent for its exemplary nutritional profile which exhibits anti- bacterial, anti-inflammatory, anti-thrombotic, anti-cancer and anti-arrhythmic properties. It is well known for its hydroxyl radical scavenging and anti-oxidant activity because of its high cysteine and methionine contents [5]. It is proven to have a rich source ω-3 PUFA which functions as modulators of cell signalling, gene expression and inflammatory processes [6, 15]. Hence today, science is finally able to provide strong evidence to support King Charlemagne beliefs.

Structure of Flaxseed ($C_{26}H_{38}O_{12}$)
Scientists from Harvard reported that ω -3 PUFAs may improve periodontal outcomes in patients with periodontitis [7]. Literature studies demonstrated flaxseed possessed anti-microbial property against various oral pathogens [17-20]. But till date, there are no studies manifesting such property against periodontal pathogens. Hence the present study was carried out to assess the *in vitro* anti-bacterial property of bioactive superfood flaxseed extract against periodontal pathogens *(Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans* and *Tannerella forsythia)* and to ascertain the effective dosage.

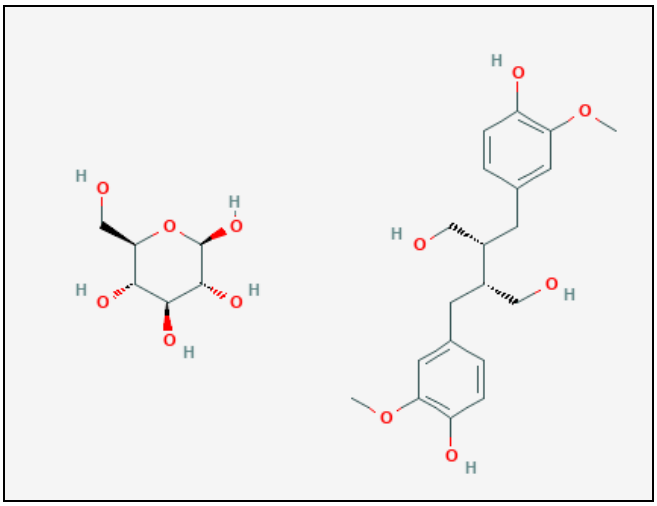

Fig 1: Structure of Flaxseed ($C_{26}H_{38}O_{12}$)

2. Materials and Methods

The study employed an *in vitro* experimental design. Ethical clearance was obtained from institutional ethical committee of Bapuji Dental College and Hospital, Davangere with Ref. No. BDC Exam/291/2018-19

2.1 Preparation of extract from flaxseed

The flaxseed extract was obtained from Bapuji Pharmacy College, Davangere. Flaxseeds were grinded until a homogenous powder was obtained (110–120 mesh). The powder was defatted with *n*-hexane (1: 6 w/v) at room temperature for 16 hours and was macerated with 100% ethanol for 3 days. The alcoholic decoction was subjected to filtration with Whatman #1 filter paper to obtain a clear filtrate of ethanolic extract, approximately 15ml [7]. The filtrate thus obtained was sent to Maratha Mandal central research laboratory, Belgaum for evaluation of anti-microbial activity against periodontal pathogens (*Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans and Tannerella forsythia).*

2.2. Periodontopathogens and stock culture

A stock culture of periodontal pathogens mainly *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans and Tannerella forsythia* was prepared. Media and reagents used were Himedia M210 (500gm) and Brain Heart Infusion(BHI) broth 500gm, Calf brain infusion 200.00 gm/L, Beef heart infusion 250.00gm/L, Proteose peptone 10.00gm/L, Dextrose 2.00gm/L, Sodium chloride 5.00gm/L, Disodium phosphate 2.50gm/L with Final pH (at 25 ºC) 7.4+/- 0.2. For facultative anaerobes, tubes were incubated at 37 °C for 48-72 hrs in Co_2 Jar. For strict anaerobes, tubes were incubated in anaerobic jars for 48-72 hrs.

2.3 Minimum Inhibitory Concentration

MIC was obtained by Thioglycollate broth. Test tubes were labelled from 1 to 10 followed by serial dilution of flaxseed extract at concentrations of 100 microlitre/ml, 50, 25, 12.5, 6.25, 3.12, 1.6, 0.8, 0.4 and 0.2 microlitre/ml respectively. In the initial tube 20 microlitre of drug was added into the 380 microlitre of thioglycollate broth. For dilutions 200 microlitre of thioglycollate broth was added into the next 9 tubes separately. Then from the initial tube 200 microlitre was transferred to the first tube containing 200 microlitre of thioglycollate broth. This was considered as 10^{-1} dilution. From 10^{-1} diluted tube 200 microlitre was transferred to second tube to make 10^{-2} dilution. The serial dilution was repeated up to 10^{-9} dilution for each drug. From the maintained stock cultures of required organisms, 5 microlitre was taken and added into 2ml of thioglycollate broth. In each serially diluted tube 200 microlitre of above culture suspension was added and were incubated for 48-72 hours in anaerobic jar at 37 °C and observed for turbidity [9].

2.4 Minimum Bactericidal Concentration

To determine MBC, from the MIC dilutions tubes, first 3 or 5 tubes were plated (which was sensitive in MIC) and incubated for 24 hours then next day the colony count was taken. MBC was carried out to evaluate the bacteriostatic or bactericidal effect.

3. Results and Discussion

3.1 Minimum Inhibitory Concentration

Table 1: R- Resistant (Bacterostatic), S- Sensitive

Sl. No.	Samples	100 p1/m1	50 p1/m1	25 p1/m1	12.5 p1/m1	6.25 0/ml	3.12 pliml	1.6 p1/m1	0.8 pliml	0.40 ml	0.2 p1/m1
	Flaxseed extract										
1	Pg	S	R	R	R	R	R	RR		R	R
2	Tf	S	R	R	R	R	R	RR		R	R
3	Aa	S	R	R	R	R	R	RR		R	R

Fig 2: Serial dilutions for *P. gingivalis*

The MIC of flaxseed extract demonstrated that the periodontal pathogens such as *P. gingivalis, A. actinomycetemcomitans and T. forsythia were* sensitive at the concentration of 100 microlitre/ml.

3.1.2 Minimum Bactericidal Concentration

Table 2: NG-No Growth (Bactericidal) Reducing number of the colonies-indicate a partial or slow bactericidal activity.

SI. No.	Samples	100 ! AAA	50 µl/nil	25 p1/m1	12.5 ti1/m1	6.25 u1 nil	3.12 ttl/ml	1.6 g1/m1	0.8 it1/m1	0.4 g1/m1	0.2 pliml
extract	Flaxseed										
1	Pg	NG	48	69	82	85	92	118	126	152	160
2	Tf	38	40	68	70	98	102	117	128	156	280
3	Aa	52	60	78	89	91	112	178	208	298	300

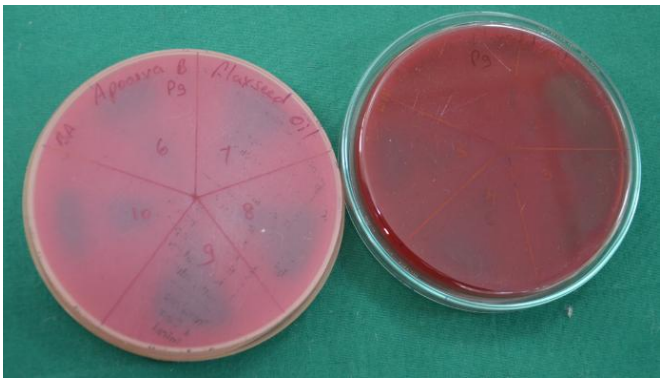

Fig 3: MBC of *P. gingivalis*

The MBC showed no growth against *P. gingivalis* at100 microlitre/ml. However, as the concentration of flaxseed extract increased, the number of *A. actinomycetemcomitans and T. forsythia* reduced in their count.

Considerable studies revealed a strong positive correlation between the presence of *P. gingivalis* and periodontal disease [10, 11]. *P. gingivalis* is a Gram-negative anaerobic bacterium, producing an innumerable virulence factors that causes tissue destruction by modulating the host inflammatory response and is considered as prime etiologic agent in chronic periodontitis. On bacteria-host cell interaction, *P. gingivalis* induces cellular signalling alteration in dental plaque and in particular cysteine like protease activity which is closely related with the clinical markers of periodontal disease. Gingipains and other virulence factors produced by *P. gingivalis* are responsible for about 85% of total host protein degradation activity [12]. Hence it is a keystone pathogen where the bacteria stimulate the aggregation of polymorphonuclear leukocytes at the gingival site and sequential release of proteolytic enzymes and reactive oxygen species (ROS) leading to destruction of periodontium [13, 14]. Further a synergistic association of *A. actinomycetemcomitans*, *T. forsythia along with P. gingivalis* are the part of mixed microbial community causing detrimental effects on the periodontium by inducing the production of leukotoxins and trypsin like proteases [16]

To terminate this process of periodontal disease, various therapies have been exploring. Hence, present *in vitro* study is stepping ahead with the usage of flaxseed. The observations from our study demonstrated that the flax extract to be bactericidal against *P. gingivalis at 100* microlitre/ml. This suggests that at this concentration it can be used as potential therapeutic agent. Current work was compared with existing literature where Secoisolariciresinol diglucoside (SDG) an important lignan found in hull of flaxseed possessed anti-microbial activity against the bacterial species where *E. coli* was inhibited at 100 ppm and it also inhibited the growth of *S. aureus* and *A. tumefaciens* at 150 ppm. *P. aeruginosa* and *B. cereus* at 200 and 300 ppm respectively [8, 17]. Further, flaxseed oil significantly lower the biofilm thickness and reduced the bacterial count against Methicillin resistant *Staphylococcus aureus* (MRSA), Methicillin sensitive *Staphylococcus aureus* (MSSA), *Klebsiella pneuminae* and *Staphylococcus epidermidis* [18].

Recently the efficacy of flaxseed was tested by measuring the zone of inhibition where the antibacterial activities were compared with streptomycin as positive control and DMSO as negative control against *Streptococcus mutans, Streptococcus pyogenes, P seudomona aerigenosa* and concluded that flaxseed extract has antibacterial activity against these selective oral pathogens [19].

Observations from literature and from the present *in vitro* study it can be hypothesized that the antibacterial property of flaxseed is probably due to lignans, phenolic acid and flavonoids [20] manifested in bactericidal activity against *P. gingivalis*.

4. Conclusion

Customary herbal medicament plays a substantial role in contemporary universe. Till date there are no *in vitro* studies to assess the antibacterial activity of flaxseed against periodontal pathogens. Present study demonstrated that flaxseed extract to be efficacious against *P. gingivalis,* which substantiated its bactericidal potency and based on these results interventional approach shall be constituted to devise an *in vivo* study further, to assess the favourable effects of flaxseed extract in chronic periodontitis patients.

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