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Evaluation of Antibacterial Activity of Bacopa Monnieri Extract on Periodontogenic Bacteria– An in-Vitro Study

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Abstract: The aim of the study was to determine the antibacterial activity of pure Bacopa monnieri extract on periodontopathogenic bacteria; Porphyromonas gingivalis(Pg), Prevotella intermedia (Pi), Fusobacteria nucleatum (Fn) & Aggregatibacter actinomycetemcomitans (Aa). The minimum inhibitory concentration (MIC), minimum bactericidal concentrations (MBC) and Time kill curve assay were performed to assess the antibacterial effect of both ethanolic and aqueous extract of pure bacopa monnieri against periodontopathogenic bacteria by serial dilution method and colony forming units respectively. MIC values of ethanolic extract were in a range of 25-100 µg/ml for Pi, Fn and Pg and Aa showed a value of 0.8 µg/ml whereas the MIC values of aqueous extracts were in a range of 50-100 µg/ml and that of Aa was 0.4 µg/ml. The time kill curve showed a fast and sharp antibacterial activity of the ethanolic extract over Pi, Fn and Aa at baseline (0 min) whereas Pg showed no growth of colonies at 2 hours.The ethanolic and aqueous extract of Bacopa monnieri exhibited durable antibacterial activity on common periodontogenic bacteria.

Keywords: Periodontal pathogens**,** Bacopa monnieri, Antibacterial activity, MIC, MBC, and Time kill curve assay

INTRODUCTION

Periodontitis is an inflammatory disease that causes destruction of tooth supporting tissues and is characterized by multifactorial aetiology with pathogenic bacteria being the primary causative agent that dwells the subgingival area[1]. Among the pathogens, Gram negative bacteria, predominantly Porphyromonas gingivalis (Pg), Prevotella intermedia (Pi), Fusobacterium nucleatum (Fn), and Aggregatibacter actinomycetemcomitans (Aa) are strongly implicated in the aetiology of chronic periodontitis[2].

The primary objective of standard periodontal therapy is to reduce the total bacterial load and to change the environmental conditions which prevent the growth and multiplication of the microbes[3]. Although scaling and root planing reduces the level of subgingival bacteria, it does not eliminate all the pathogens which reside deep in the connective tissue which could later be responsible for tissue destruction [3]. One out of many reasons for incomplete elimination of pathogens could be the complex anatomy of the root and the location of the lesion which may hamper the treatment and prevent sufficient reduction of the bacterial load [4]. To overcome this limitation, systemic antibiotics are used along with mechanical debridement in the management of periodontal infection. However, systemic administration of antimicrobials has been reported to cause the development of multi-resistant microorganisms, inter bacterial transfer of resistance determinants, and side effects. Thus to combat this global issue, an effective and economical solution has to be discovered.

Herbal plants have been used as traditional treatment for numerous human diseases for thousands of years, as plant based drugs are biodegradable, safe and have fewer side effects. Many plants with biological and antimicrobial properties have been studied since there has been a relevant increase in the incidence of antibiotic overuse and misuse [5].

Bacopa monnieri commonly known as Brahmi (synonyms: Lysimachia monnieri L. Cent., Gratiola monnieri L., Monniera cuneifolia and Herpestis monniera L.) [6] (Table 1) is a thyme leaved gratiola, belong to the scrophulariaceae family. It is well known as a memory vitalizer and is still used in traditional

medicine to treat various nervous disorders and to provide relief to patients with anxiety. Brahmi is also used in digestive complains, skin disorders, and as an antiepileptic, antipyretic, and analgesic [7]. This plant also displays additional medicinal characteristics like cardiovascular and smooth muscle relaxant effect. Studies have also proved the antimicrobial efficiency of the herb on various microbial pathogens [8]. The numerous pharmacological properties of the herb can be attributed to the presence of various phytochemicals present in it (Table 2). Apart from saponins, which are the predominant phytochemical present, other active compounds responsible for the pharmacological effects of Bacopa monnieri include alkaloids, sterols, Brahmine, herpestine, tannins and flavonoids. More recently novel saponins called bacopasides I–XII has also been identified [9-14].

To the best of our knowledge, no study has been done to assess the antibacterial effect of Bacopa monnieri extract on the most common periodontal pathogens. Hence, the aim of the present study was to assess the minimum inhibitory concentrations (MICs), minimum bactericidal concentration (MBC) and time killed curve essay of pure Bacopa monnieri extract that can be safely and effectively administered as antibacterial agent on periodontal pathogens.

MATERIALS AND METHODS

Preparation of Alcoholic and Aqueous extracts

About 100% pure Bacopa monnieri crude powders (300 mg) were obtained from Vyas Pharmaceuticals, Indore. It was certified to be free from any form of bacteria, yeast, or mold, by the manufacturer after microbial analysis. The alcoholic and aqueous extracts were prepared using the maceration technique, wherein 150 gm of coarsely powdered crude compound is macerated with 500 ml alcohol solvent for 24 hours. The same procedure was carried out for the preparation of aqueous extract, in which the crude powder was macerated with 500 ml of with distilled water for 24 hours.

BACTERIAL STRAINS

Bacterial strains used in this study were American type culture collection, Manassas, VA, USA. The tested bacterial strains in this study were Pg, ATCC 33277, Pi ATCC 25611, Fn, ATCC 25586 and Aa, ATCC 29523.

ANTIBACTERIAL ACTIVITY

Minimum inhibitory concentrations

MIC is the lowest concentration of antimicrobial agent that will inhibit visible growth of bacteria after overnight incubation [15]. Stock solution of the antimicrobial agent was prepared by adding 100 µg of the extract to 1 ml of Thioglycollate (TG) broth medium (100 µg/1 ml). For MIC, nine dilutions of the drug were prepared with TG broth medium using microdilution method by means of standard protocols[15]**.** The minimum concentration of the drug in the tube which does not show any turbidity is considered as the MIC of the drug.

Minimum bactericidal concentration

MBC is the lowest concentration of antimicrobial agent that will prevent the growth of bacteria after sub-culturing on to antibiotic free media.[15] After the MIC procedure, all the 10 dilution tubes were taken and inoculated into respective culture medium to check the growth of microorganisms. Formerly plates were incubated in anaerobic jar/chamber for ≥48 h and then colonies were counted.

Time Kill Curve Assay

Time-kill curve assay is the most appropriate method for defining the bactericidal outcome and is an efficient tool for obtaining evidence about the interaction between the anti- microbial agent and the microbial strain. The assay was performed in broth culture medium using 4 test tubes containing a bacterial suspension of equal quantity of broth and compound. The suspension was then shifted into 4 different tubes and these were cultured at different time intervals (5mins, 10mins, 30mins and after 2hrs). At the end of plating, these tubes were then incubated according to the growth requirement; that is in CO_2 jar and anaerobic jar. After 48-72hrs of incubation the plates were removed and the percentage of dead cells is calculated relatively to the growth control by determining the number of living cells (CFU/mL) of each tube using the agar plate count method.

RESULTS

The results showed, ethanolic extract exhibited a MIC value of 0.8 µg/ml and aqueous extract at low concentrations. The MIC value of ethanolic and aqueous extract to restrict the growth of *A.a* was found to be 0.8 µg/ml and 0.4 µg/ml respectively (Table 3). While *Pi*, *Fn* and *Pg* showed MIC values of 25 µg/ml, 50 µg/ml and 100 µg/ml for ethanolic extract and 50 µg/ml, 100 µg/ml and 100 µg/ml for aqueous extract respectively. From the MBC values it was deduced that there was a growth detected for all the organisms at all concentrations (Table 4). The time kill curve for the ethanolic extract displayed no growth for *Pi*, *Fn* and *Aa* from baseline to 2 hours whereas *Pg* showed no growth within 2 hours of plating. The aqueous extracts showed no growth of all the four organisms within a time interval of 2 hours (Graph 1).

Table−1: Other common traditional and scientific synonyms of Bacopa monnieri.

Synonyms of Bacopa monnieri	Bacopa monniera
	Herpestis monniera
	water hyssop
	thyme leaved gratiola
	Brahmi
	Jal Neem bootee

Table−2: Chemical composition of Bacopa monnieri

Chemical constituents		**Properties**
Alkaloids	1. Brahmine 2. Nicotinine 3. Herpestine 4. Monnierin	• Act on nervous system particularly on the action of chemical transmitters. • Antihypertensive, antiarrhythmic effect, antimalarial and anticancerous.
Saponins	✓ Triterpenoid saponins. ✓ Saponins A, B and C. ✓ Bacosides A [3-(α-L-arabinopyranosyl)-O-β-Dglucopyranoside-10,20-dihydroxy-16-keto-dammar-24-ene.	• Bacosides A and B improve the transmission of impulses between the neurons. Bacosides regenerate synapses and repair damaged neurons. • Responsible for the cognitive effect. • Scavenging capacity on free radicals. • Antioxidant, anticancer, anti-inflammatory and antifungal action
Tannins	-	• antiviral, antibacterial and anti-tumour activities,
Flavanoids	-	• Ability to modify the body's reaction to allergies and virus. • Anti-allergic, anti-inflammatory, anti-microbial and anti-cancer activities.
Other constituents	✓ Betulinic acid, ✓ D-mannitol, ✓ Stigmastanol , ✓ β-sitosterol ✓ Stigmasterol	• Antifungal property • Other properties of these constituents are under research

Table−3: MIC values of ethanolic extract

Bacteria	100µg/ml	50	25	12.5	6.25	3.12	1.6	0.8	0.4	0.2
Fn	S	S	R	R	R	R	R	R	R	R
Pg	S	R	R	R	R	R	R	R	R	R
Pi	S	S	S	R	R	R	R	R	R	R
Aa	S	S	S	S	S	S	S	S	R	R

Table−4: MBC values of ethanolic extract (The values are the number of viable cells present after incubation)

Bacteria	100 µg/ml	50 µg/ml	25 µg/ml	12.5 µg/ml	6.25 µg/ml	3.12 µg/ml	1.6 µg/ml	0.8 µg/ml	0.4 µg/ml	0.2 µg/ml
Fn	74	83	90	102	104	114	116	123	128	138
Pi	76	91	94	96	108	119	203	205	211	213
Pg	68	70	81	88	98	110	112	118	122	130
Aa	38	42	49	58	69	72	74	81	84	86

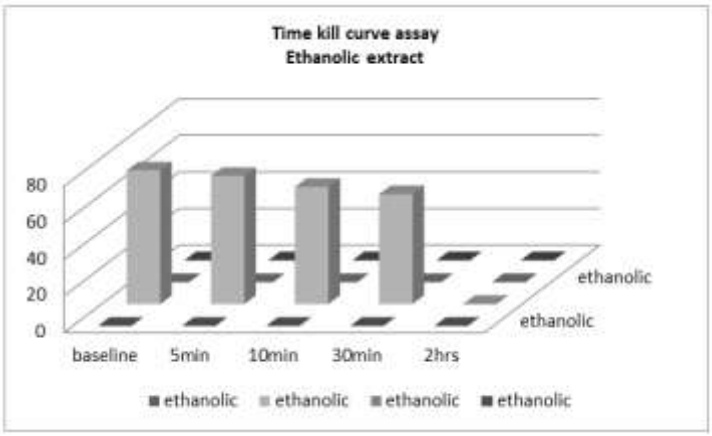

Fig-1: Graphical representation of Time kill curve of Ethanolic extract of Bacopa monnieri.

DISCUSSION

Traditional medicine is being used since times immemorial, for their pharmacological applications like antiulcerogenic, wound healing, anti-inflammatory, antimicrobial, antioxidant properties etc [16]. The pharmacological properties of the herbal medicines are due to the presence of certain phytochemicals in them, which offer an effective alternative to antibiotics and represent a promising approach in the prevention and therapeutic strategies for oral infections.

Bacopa monnieri, or Medhya Rasayana[12]**,** is one of the greatest versatile herb which is extensively investigated for its pharmacological and therapeutic effects. This medicinal plant is a great immune-stimulant, tranquilizer, mind pacifier, neuroleptic, psychotropic as well as an anti-inflammatory [7], antioxidant [7] antiarthritic [17] and antifungal [18]**.** The properties that the herb possess has been attributed to the presence of different types of saponins such as bacosides A, B, C and D which are the active triterpenoid principles "memory chemicals"[12]**.**

Studies on the antimicrobial properties of Bacopa monnieri are limited in literature and so this is the first study which determines the in-vitro antibacterial activity of ethanolic and aqueous extracts of Bacopa monnieri against common periodontopathogenic bacteria, by MIC, MBC and Time kill method.

Sampathkumar *et al*. assessed the antibacterial potency of diethyl ether and ethanolic extract of Bacopa monnieri against gram positive organism like *S.aureus and Proteus vulgaris* and found that the ethanolic extract showed more of anti-fungal activity [19] than antibacterial property, which is contradictory to the results obtained in the present study, wherein it was observed that the ethanolic extract do possess an antibacterial property against gram negative organisms. Pawar et al in their study observed that both methanol and aqueous extracts of Bacopa monnieri did not project any significant antimicrobial property when subjected to bacterial strains like *Staphylococus aureus, Bacillus subtilis, Escherichia coli and Pseudomonas aeruginosa* [20]. But this was again contradictory to the results obtained in the present study which state that both aqueous and alcohol extract do possess an antibacterial property against gram negative strains. The antibacterial action of methanol extract of Bacopa monnieri was assessed by Mathur *et al*. and observed that the alcoholic extract possess a strong antimicrobial property when compared to the aqueous extract which showed minimum activities against Gram positive organisms [21]. The observation made by Mathur *et al*. was correlating hand in hand with the results obtained in the present study. Similar observations were made by Khan *et al*. who assessed for the antibacterial action of Bacopa monnieri leaf extracts against 7 Gram positive and 11 Gram negative bacteria using different solvents which showed an agreement with the results obtained in the present study [22].

The present study exhibited a far-reaching extremity in the MIC and MBC values of both ethanolic and aqueous extracts. The range of MIC values of ethanolic extract of Bacopa monnieri is from 100-0.8 µg/ml with the highest value noted for *P. gingivalis* and the lowest value noted for *A. actinomycetemcomitans*. The MBC value of ethanolic extract was found to be least in A. actinomycetemcomitans whereas the highest MBC value of aqueous extract was found for *F.nucleatum*. Thus, suggesting that the ethanolic extract has a greater bacteriostatic activity against *A. actinomycetemcomitans*[23]. Hence, from this study, it can be inferred that *A. actinomycetemcomitans* was sensitive to both ethanolic and aqueous extracts.

The enhanced antibacterial action of ethanolic extract of Bacopa monnieri could be due to the presence of various phytochemicals like tannins, flavonoids, saponins and alkaloids which possess certain basic character that allows them to react with proteins to form stable water soluble compounds thereby killing the bacteria by directly damaging its cell membrane. It can also be considered that the pattern of inhibition varied

with the plant extract and the organism tested. At the same time, the decreased antimicrobial action of the aqueous extract can be attributed due to the loss of some of the active compounds during the extraction process of the sample[19,20].

The time-kill test reveals a time-dependent or a concentration-dependent antimicrobial effect. The test showed that the ethanolic extract had a potent bactericidal effect over *P.intermedia*, *F.nucleatum* and *A.actinomycetemcomitans* within baseline (0 mins) whereas the extract showed a bactericidal activity against *P.gingivalis* within 2 hours. The marked bactericidal property of ethanolic extract could be due to the crude concentration of the extract used to perform the time kill procedure. The above results help us infer that Bacopa monnieri extract has a concentration dependent killing property [24]. The time kill test presented the bacteriostatic property of the extracts [23].

CONCLUSION

From the present study it can be concluded that Bacopa monnieri has a potent antimicrobial activity over the common periodontal pathogens. Both the ethanolic and aqueous extract showed improved antibacterial activity, yet the ethanolic extract displayed an amplified bacteriostatic property when compared to aqueous extract. Thus further studies may be required to check up on the enhanced antimicrobial activity of the herb with higher concentrations and to use the properties of the plant efficiently as an adjunct to routine periodontal therapy as a local drug delivery system (gel, chip, buccal patches or mouthwash) in meritoriously treating periodontal diseases.

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