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IN- VITRO ANTIBACTERIAL ACTIVITY OF SPILANTHES ACMELLA (AKARKARA) EXTRACT ON *PORPHYROMONAS GINGIVALIS* AND *AGGREGATIBACTOR ACTINOMYCETEMCOMITANS*

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ABSTRACT

Periodontal diseases are caused by certain bacteria found in the bacterial plaque. Usage of plant-derived antimicrobial agents could serve as an effective alternative treatment against periodontal infections due to continuous rise seen in antibiotic resistance. *Spilanthes acmella* (*S. acmella*), a vital medicinal plant has been used for its various properties such as anti- inflammatory, antibacterial, antifungal, antinociceptive, anti-cancerous and hastening wound healing. This study was conducted to assess its antibacterial efficacy against common periodontal pathogens.

Objective: The present study was conducted to assess the antibacterial activity of S. *acmella* plant extract against *Porphyromonas gingivalis (Pg)*, and *Aggregatibactor actinomycetemcomitans* (Aa) and determine the presence of various phytochemicals in it.

Materials and Methodology: An extract was prepared using dried S. *acmella* plant powder and mixed with methanol in 1:1 (w/v) ratio. Determination of minimal inhibitory concentration (MIC) was done by using tube dilution technique and time- kill assay was performed against *Pg* and *Aa*. Presence of phytochemicals was checked by thin layer chromatography (TLC) method.

Results: MIC of S. *acmella* was found to be 40 µg/ml for *Pg* and 20 µg/ml for *Aa* within 2 h interval. Various phytochemicals were found in S. *acmella* extract which may be responsible for its anti- bacterial property.

Conclusion: S. *acmella* extract shows a significant antibacterial effect against the major periodontal pathogens and hence may be a potential natural alternative for controlling the growth of these bacteria.

Keywords: *Spilanthes Acmella, Porphyromonas Gingivalis, Aggregatibactor Actinomycetemcomitans,* Minimal Inhibitory Concentration, Time Kill Assay

1. INTRODUCTION

Oral health is crucial for general health and the quality of life of the individual. Vlachojannis et al. (2018) According to World Health Organization (WHO) 80 percentage of the world's population presently uses herbal medicines for some aspect of primary health care. World Health Organization (2011) Natural products have been used since decades for various purposes. Today plants are being used in

maintaining health, diagnosis, prevention and in the treatment of physical illness such as arthritis, kidney diseases, migraine, allergies, skin diseases, wounds, burns, gastrointestinal issues and even cancer as well as in mental illness. Paulraj et al. (2013)

Periodontitis is defined as an inflammatory disease of supporting tissues of teeth caused by specific microorganisms resulting in progressive destruction of the periodontal ligament and alveolar bone with periodontal pocket formation, gingival recession, or both. Newman et al. (2006) Periodontal pockets accommodate a number of bacterial phylotypes including commensals and true pathogens. *Porphyromonas gingivalis (Pg)* and *Aggregatibactor actinomycetemcomitans (Aa)* have been detected in large numbers in periodontal pocket. Three species, *Pg, Aa* and *Bacteriodes forsythus* were strongly associated with progressive status of the periodontal disease and unsuccessful therapy. Socransky & Haffajee (2002) Scaling and root planing (SRP) is considered as the gold standard for the treatment of periodontitis. Newman et al. (2006) However, treated sites are subjected to recolonization within few months with a microbiota similar to that present before therapy. Mombelli (2018) So, adjuvant therapies such as antibiotic therapy are mainly used to combat such microbes but due to their misuse, microbes develop increased resistance to many common antibiotics. Vlachojannis et al. (2018) Synthetic antiseptics are currently in use to reduce the bacterial load and chlorhexidine (CHX) is considered as the gold standard in dentistry. Vlachojannis et al. (2018) However, there are drawbacks to the use of CHX which includes staining of teeth and mucosa, dysgeusia, as well as long term usage of CHX may lead to the emergence of new resistant staphylococci strains. Vlachojannis et al. (2018) In light of the growing antibiotic resistance, the usage of plant-derived antimicrobial agents could serve as an effective alternative treatment against oral infections.

The genus *Spilanthes* belongs to the family Asteraceae also known as family Compositae which is comprised of more than 300 species. Paulraj et al. (2013) Different species in this genus are found to have a wide range of therapeutic and medicinal properties such as hepatoprotective and diuretic properties. Paulraj et al. (2013) *Spilanthes acmella* (*S. acmella)* which is also known as eyeball plant, spot plant and Para cress, is a vital medicinal plant prominently distributed in the tropical and subtropical regions around the world including India. Abdul Rahim et al. (2021) These plants have been popularly called toothache plant, the reason being its major use for toothache where the fresh flower head and/or leaves are chewed or placed in the cavities of decayed teeth which relieves the pain with its anesthetising property. Abdul Rahim et al. (2021) All parts of the plant are bitter in taste, with the flower heads being the most pungent part which on consuming causes a tingling sensation, numbness, and excess salivation. Abdul Rahim et al. (2021)

S. acmella is known to be a rich source of important bioactive compounds and these bioactive compounds have been used for its various properties such as anti-inflammatory, antibacterial, antifungal, antinociceptive, anti-cancer activities, and promote wound healing. Prachayasittikul et al. (2013) Spilanthol, which is *N*-isobutylamide, is the major phytochemical present in *S. acmella* which is responsible for its various biological activities. Yasuda et al. (1980) *S. acmella* offers active metabolites called phenolics, including as vanillic acid, trans-ferulic acid, trans-isoferulic acid, and stigmasteryl glucoside, which are highly effective antioxidants. Prachayasittikul et al. (2009) The bioactive substances from each part of *S. acmella* have been shown in the literature to have exceptional pharmacological activity. Prachayasittikul et al. (2009)

To the investigator's knowledge, literature pertaining to the antimicrobial activity of *S. acmella* on periodontal pathogens has not been reported earlier. Therefore, the present study was conceptualized as an initial step to evaluate the minimal inhibitory concentration of *S. acmella* against common periodontal pathogens.

In this in vitro study, *Spilanthes acmella* extract was evaluated for,

- To assess the antibacterial activity of *S. acmella* plant extract against *Porphyromonas gingivalis (Pg),* and *Aggregatibactor actinomycetemcomitans(Aa)*.
- To determine the presence of various phytochemicals.

2. MATERIALS AND METHODS

2.1. PREPARATION OF EXTRACT

S. acmella plants were obtained from local dealer under aseptic conditions and the specimens were identified by a botanist for their authenticity. Flowerheads, leaves, stems and roots were seperated from the plant, washed in distilled water and dried in the sun for a week. They were then blended using electric blender to obtain a fine powder. Methanol extract of *S. acmella* was prepared in 1:1 (w/v) ratio using Soxhlet apparatus. Charu et al. (2022) 50g of powdered sample was filled into a thimble and subjected to Soxhlet extraction using 150ml of 99% methanol as solvent. The extract was concentrated using rotary evaporator and placed in incubator for 24 h at room temperature. After 24 hrs, the mixtures were filtered through 8 layered muslin cloth filter and centrifuged at 5000 rpm for 15 min. Charu et al. (2022) The supernatants were collected and the solvents were evaporated to make the final volume one-fourth of the original in a rotary evaporator at 4 rpm, 75 torr, and 50°C. Charu et al. (2022) Then the extracts were stored at 4° C in airtight bottles for further use. Charu et al. (2022) [Figure 1(a), 1(b), 1(c) and 1(d)]

Figure 1

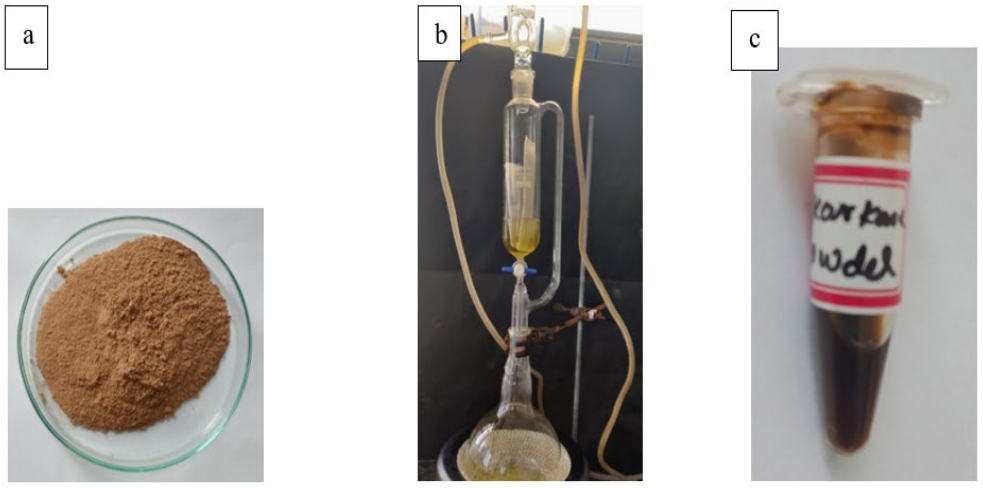

Figure 1 Extraction of Spilanthes Acmella Plant: (a) Powder of the Whole Plant (b) Soxhlet Apparatus (c) Spilanthes Acmella Plant Extract

2.2. MINIMAL INHIBITORY CONCENTRATION

Minimum inhibitory concentration (MIC) of this extract was estimated using serial dilutions of the agent by tube dilution method. Respective strains of *Pg and Aa*

were chosen for the study [Table 1]. The tubes were incubated for 24 h at 37°C. The optical density of each tube was evaluated after incubation using a spectrophotometer (Labman, India) at 600 nm at 37°C for 24 hours. Chaiya et al. (2013) The minimum concentration that repressed 100% growth of *Pg and Aa* was indicated as MIC. Briefly, concentrations from 10 µg/ml to 640 µg/ml of extract was added into the tubes containing 300 µL of thioglycollate broth following the method used by Chaiya et al. (2013) From the maintained stock cultures of *Pg* and *Aa*, 100 µL was taken and added into 2 ml of thioglycollate broth. In each serially diluted tube 100 µL of above culture suspension was added.[12] The tubes were incubated for 48–72 h in an anaerobic condition at 37°C and observed for turbidity. Chaiya et al. (2013) The respective samples were tested for Optical density at 600nm and tabulated. [Figure 2 (a) and (b)]

Table 1

Table 1 Strains Used in the Study

S. No	Test Organisms	Strain
1	*Porphyromonas gingivalis*	ATCC33277
2	*Aggregatibacter actinomycetemcomitans*	ATCC29522

Figure 2

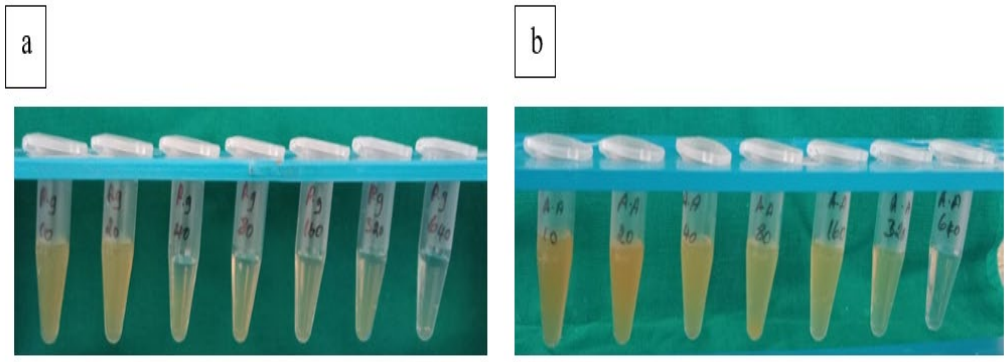

Figure 2 Serial Dilutions of Spilanthes Acmella Extract (a) Porphyromonas Gingivalis, (b) *Aggregatibacter Actinomycetemcomitans*

2.3. TIME KILL ASSAY

Following the MIC results, equal quantity of the broth with organisms (*Pg* and *Aa*) and plant extract was mixed which was then plated immediately and this was noted as 0 h [control tubes]. Appiah et al. (2017) Tubes were kept in anaerobic conditions till further time slot, that is, 10 min, 30 min, and 2 h. It was cultured and incubated according to the growth requirement. Appiah et al. (2017) After 48–72 h of incubation, the plates were removed and the number of colonies was noted Appiah et al. (2017) [Figure 3 and Figure 4].

Figure 3

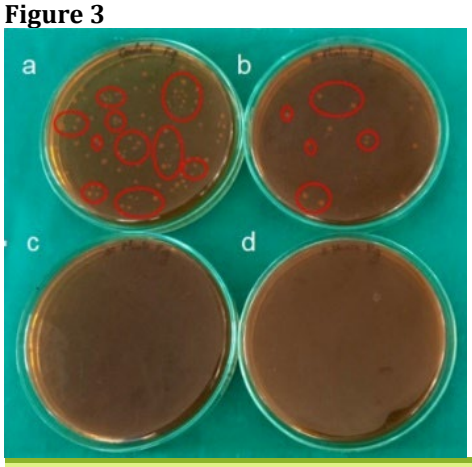

Figure 3 Time Kill Assay- Colonies of P. Gingivalis (a) at Baseline (b) after 10 min (c) after 30 min (d) after 2 hrs

Figure 4

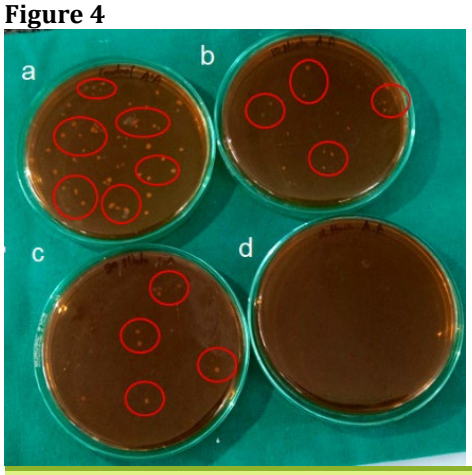

Figure 4 Time Kill Assay- Colonies of A. Actinomycetemcomitans (a) at Baseline (b) after 10 min (c) after 30 min (d) after 2 hrs

Figure 5

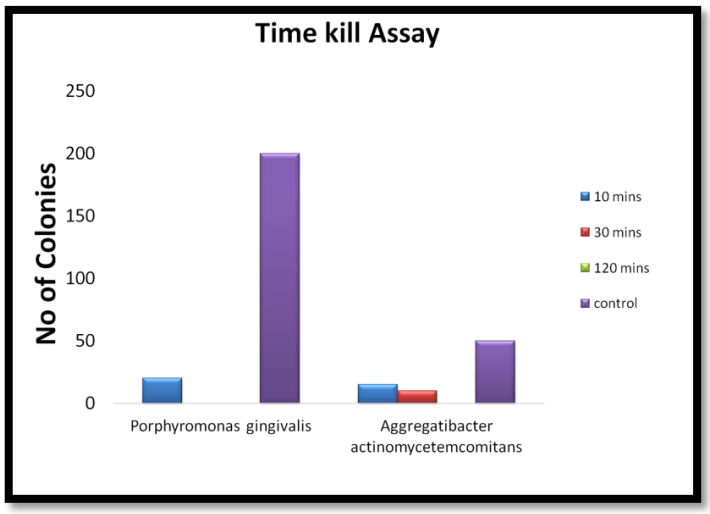

Figure 5 Time Kill Assay- Graph

2.4. PHYTOCHEMICAL ANALYSIS

TLC analysis of plant extract was done and checked for the presence of various phytochemicals [Table 2]. It was performed on 20 cm x 10 cm silica gel aluminium plate. Mian et al. (2019) Two micro liters of the extract was deposited in a glass twin-trough chamber previously saturated with mobile phase vapour for 20 min and hexane: ethyl acetate: formic acid in the ratio of 7:10:0.1 (v/v/v) was used as mobile phase. Mian et al. (2019) After development, the plates were dried with a hair dryer and then the spotted TLC plates visualized in the day light and under the UV wavelength (short & long) i.e. 254 nm, 366 nm respectively. Mian et al. (2019)

The R_f value (retardation factor) of spots was determined by the given formulae. Mian et al. (2019)

R_f Value=Distance travelled by spot / Distance travelled by solvent.

Table 2

Table 2 Various Phytochemicals Tested in the Extract

S. No	Phytochemicals
1	Alkaloid
2	Flavonoid
3	Glycoside
4	Tannin
5	Saponin
6	Steroids
7	Phenol
8	Terpenoids

3. RESULTS

In the present study, both the periodontal pathogens, namely, *Pg* and *Aa* were found sensitive to *S. acmella* methanolic extract. *Aa* was sensitive until 20 µg/ml dilution and showed resistance to further dilution. *Pg* was sensitive until 40 µg/ml dilution and showed resistance to further dilution. [Table 3 and Table 4] Time kill curve assay showed that *Pg* was inhibited within 30 min and *Aa* within 2 h [Table 5]. Various phytochemicals were found in the *S. acmella* extract i.e., Alkaloid, Flavonoid, Glycoside, Tannin, Saponin, Steroids, Phenol and Terpenoids. [Table 6].

Table 3

Table 3 MIC of Spilanthes Acmella Extract Against *Pg*

Extract Concentration (µg/ml)	MIC(µg/ml) OD at 600 nm	% Reduction *(Pg)*
10	1.433	27.03
20	1.325	32.53
40	0.756	61.50
80	0.675	65.63
160	0.243	87.62
320	0.232	88.18
640	0.213	89.15
Control (Without Sample only *Pg*)	1.964	

(MIC: minimal inhibitory concentration, *Pg*: Porphyromonas gingivalis)

Table 4

Table 4 MIC of Spilanthes Acmella Extract Against *Aa*		
Concentration (µg/ml)	**MIC (µg/ml) OD at 600 nm**	**% Reduction *(Aa)***
10	0.997	44.45
20	0.806	55.09
40	0.456	74.59
80	0.302	83.17
160	0.156	91.31
320	0.155	91.36
640	0.145	91.92
Control (Without Sample only *Aa*)	1.795	

(MIC: minimal inhibitory concentration, *Aa: Aggregatibactor actinomycetemcomitans*)

Table 5

Table 5 Time Kill Assay				
Time Kill Assay				
Organism	**No. of colonies seen after**			
	10 min	**30 min**	**120 min**	**No. of colonies in positive Control**
Porphyromonas gingivalis	20	0	0	2×10^2
Aggregatibacter actinomycetemcomitans	15	10	0	0.5×10^2

Table 6

Table 6 Various Phytochemicals in the Extract with their Percentage		
S. No	**Phytochemicals**	**Methanol Extract (%)**
1	Alkaloid	0.76
2	Flavonoid	0.7
3	Glycoside	0.84
4	Tannin	0.6
5	Saponin	0.98
6	Steroids	0.7
7	Phenol	0.73
8	Terpenoids	0.62

4. DISCUSSION

As far as the investigator is aware, there hasn't been any prior reporting of literature on *S. acmella's* antimicrobial efficacy against periodontal pathogens. Since no direct tests have been conducted, the current study's goal was to ascertain the antimicrobial efficacy of the extract against *Pg* and *Aa* using tube dilution method and time kill curve and the presence of various phytochemicals in the *S. acmella* whole plant extract. Our data confirm that *S. acmella* is a potent inhibitor of *Pg* as well as *Aa.* In addition, we found that at a minimum concentration of 40 µg/ml, both the periodontal pathogens were inhibited. The extract from S. acmella was shown to have a minimum inhibitory concentration of 40 µg/ml for Pg and 20 µg/ml for Aa. Both of the studied organisms did not develop within two hours, according to the time kill curve experiment.

A study done by Shobana G on Anti-bacterial efficacy of *S. acmella* on *salivary mutans Streptococci* in which it was found that 20% Methanolic extract of *S. acmella* was as efficacious as chlorhexidine as an antimicrobial agent on *salivary mutans Streptococci*. Shobana (2018) When compared to medications like Ca $(OH)_2$, Sathyaprasad et al. (2015)'s study revealed that *S. acmella* possesses remarkable antibacterial and antifungal activity against common root canal pathogens, such as Enterococcus faecalis and Candida albicans, which are responsible for recurrent endodontic failures. Sathyaprasad et al. (2015) The examined bacteria and fungus were significantly inhibited by the crude extracts of *S. acmella*. In one investigation by Ahmed S et al., the antimicrobial activities were modest against Salmonella typhi, Stapylococcus aureus, and Bacillus subtili, but they had strong antifugal activity against three fungi, namely Candida albicans, Aspergillus niger, and Sacharomyces cerevacae. Ahmed et al. (2012)

A study done by Praveen NC *et.al. Aa and Pg* were found to be sensitive to pineapple extract (bromelain) at a minimum concentration of 16.6 mg/ml and 4.15 mg/ml, respectively. Praveen et al. (2014) Another study conducted by Patra JK et.al., the crude extract and the two fractions (chloroform and hexane) of *Robinia pseudoacacia* were proved highly active in controlling *Pg.* Patra et al. (2015) According to Müller-Heupt et al. (2022), an ethanolic Azadirachta indica leaf extract had a MIC of 1024 mg/L and 256 mg/L for the acetone extract against Pg ATCC 33277. A 100% ethanolic extract of *Rheum palmatum* root showed a MIC of 4 mg/L for *Pg* ATCC 33277. The MIC of Eucalyptus globulus leaf extracts in acetone and ethanolic were determined to be 128 mg/L and 256 mg/L, respectively, against Pg. [20] Verma K et. al have done a similar study where they have used *Acmella oleracea* (similar species to *S. acmella*) in gel form clinically as a local drug delivery (LDD) and have found significant improvement in clinical parameters when combined with SRP. Verma et al. (2022)

A study done by Ramsevak et.al. showed that hexane extract of flower buds of *S. acmella* contained three *N*-isobutyl amides: spilanthol, undeca-2*E*,7*Z*,9*E*-trienoic acid isobutylamide and undeca-2*E*-en-8,10-diynoic acid isobutylamide. Ramsewak et al. (1999) Qualitative phytochemical screening of *S. acmella* extracts done by Rao TM et.al. demonstrated the existence of many phytochemical components, such as steroids, terpenoids, flavanoids, alkaloids, glycosides, tannins, carbohydrates, oils, and amino acids. Also, the methanolic extract had more phenolic content when compared to other extracts. Rao et al. (2012) Nakatani and Nagashiwa demonstrated that the existence of amides- spilanthol and alkamides that may be the cause of the antibacterial and antifungal action of various concentrations of *S. acmella* extract. Nakatani & Nagashiwa (1992) presence of nonvolatile sesquiterpenoids and saponins were also reported by Krishnaswami et al. and Mukharya et al. as potentially contributing to the antibacterial and antifungal properties of *S. acmella*. Krishnaswami et al. (1975), Mukharya & Ansari (1986)

5. CONCLUSION

The *S. acmella* extract shows a significant antibacterial effect against the major periodontal pathogens i.e. *Pg* and *Aa* and hence may be a potential natural alternative for controlling the growth of these bacteria. *S. acmella* possess various phytochemicals which could be responsible for its anti- bacterial property. In conclusion, the genus *Spilanthes* offers a wide range of research possibilities. To ascertain its therapeutic effectiveness and suitability for incorporation into regular at-home oral hygiene products, more in vivo research studies are required.

CONFLICT OF INTERESTS

None.

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