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Comparative Evaluation of Efficacy of Spilanthes Acmella Flower Extract as an Intracanal Medicament with Calcium hydroxide and 2%Chlorhexidine gel –An in Vitro Study

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Abstract
Aim- To compare and evaluate effectivity of Spilanthes Acmella flower head extract as a potent intracanal medicament with time tested calcium hydroxide and 2% chlorhexidine gel. **Materials and Methods**- The bacterial and fungal strains of Enterococcus faecalis (ATCC 29212) and Candida albicans (ATCC 10231) respectively, were tested against 5% and 10% of Spilanthes acmella flower extract and calcium hydroxide and 2% chlorhexidine gel.Fixed volumes (100 microliters) of Spilanthes acmella flower head extract in concentrations of 5% and 10%, 2% chlorhexidine gel, 5% and 10% of calcium hydroxide were then transferred by micropipettes under aseptic conditions. The agar plates were maintained at room temperature for 1hr to allow uniform spread of the solvents and then they were incubated at 37° C for up to 24-48 hrs. The zones of inhibition were measured by using mm scale and the diameter was noted.**Statistical Analysis**- Results of Spilanthes Acmella flower extract were compared with calcium hydroxide and 2% chlorhexidine gel using One-way analysis of variance (ANOVA) , Post-hoc Tukey's test, Median test and Kruskal-Wallis test. Result- 10% Spilanthes Acmella extract (SPA) showed significant zone of inhibition against E.feacalis and C.albicans in comparison to 5% and 10% calcium hydroxide. However, 2% Chlorhexidine gel showed dominance over both SPA extract and calcium hydroxide. **Conclusion**- Spilanthes acmella extract has significant antibacterial and antifungal property and has the potential to be utilized as an intracanal medicament.

Introduction
The most constitutional part of root canal treatment is to accomplish debridement of microorganisms from root canal systems. The chemo-mechanical preparation has a considerable decontaminant action but, it cannot decimate all the microorganisms from root canal systems. These unexpended bacteria proliferate throughout the period between appointments in the cases where the canal is not dressed with an intracanal medicament between visits.

In the past few decades, several intracanal medicaments ranging from phenolics to aldehydes and antibiotics have been used. Calcium hydroxide has been the gold standard in intracanal medicament and is in use since 1920's. Nonetheless, it was found to be ineffective in eliminating specific microbes such as *Enterococcus faecalis* and *Candida albicans* from root canal spaces.[1] Chlorhexidine has risen as a substantial intracanal medicament.However, studies

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have shown that chlorhexidine gel with manual or rotary instrumentation showed small amount of debris extrusion leading to post-operative pain.[2]This has led us to search for an alternative medicament that would maximize microbial eradication without the risk of causing mutagenicity and developing bacterial resistance .

There has been a boost in therapeutic importance of natural herbal extracts in dental science in the recent days. *Spilanthes acmella* commonly known as tooth ache plant, has been used as a traditional folk medicine for years to cure toothache. Although itsantibacterial, antifungal, anti-inflammatory, analgesic and anesthetic action is well documented, not many studies has been done to test its efficacy against the commonly occurring root canal pathogens and also the studies have not analogized it with calcium hydroxide and chlorhexidine gel, the two most routinely used intracanal medicament. This study thus aims at evaluating the antibacterial and antifungal effect of extract of flower *Spilanthes acmella* against the common endodonpathogens *Enterococcus faecalis* and *Candida albicans* and comparing the findings with that of calcium hydroxide and chlorhexidine gel .

Material And Methods

1. Single use disposable vial of *Enterococcus faecalis* (ATCC 29212) and *Candida albicans* (ATCC 10231) (MGM Microbiology Labs and Hospital)
2. Herbal plant of *Spilanthes acmella* will be obtained from indiamart.com
3. Soxhlet apparatus, Extraction solvent (acetone), Rotary evaporator, Dimethyl sulfoxide.(D.Y. Patil Biotechnology College, Belapur)
4. Intracanal medicaments – Calcium hydroxide powder (Prime Dental), 2 % Chlorhexidine gel (Cerkamed)
5. Brain heart infusion broth (Hi media labs) and Sabouraud's dextrose agar medium (Hi media labs)
6. 35^0 C to 37^0 C incubator, Microgram weighing machine, saline, petri dishes, filter paper, inoculating loop or needle

Preparation of herbal extract

Air dried flower heads of Spilanthes *acmella* were placed inside a thimble made from thick filter paper, which was loaded into the main chamber of the Soxhlet extractor. The extractor was placed onto a flask containing the extraction solvent, acetone ($40\text{-}60^0$C). The solvent was heated to reflux, and the vapors then traveled up a distillation arm and flooded into the chamber containing flower head, dissolving the flower heads into the warm solvent. As the Soxhlet chamber gets almost full, it is automatically emptied by a siphon arm with the solvent running back down to the distillation flask. The cycle was repeated 5-8 times, over two days and during each cycle, a portion of nonvolatile compounds dissolve into the solvent, which was eliminated by means of a rotary evaporator/ shaker (100rpm), yielding the extracted compound.

Preparation of Enterococcus *faecalis* and Candida *albicans*inoculum

The bacterium was grown and maintained on BHI agar (HiMedia) for *E.faecalis* and on SDA agar (HiMedia) for *C.albicans* from which new stock culture plates were prepared periodically & stored in the refrigerator (-20^0C).

Inoculation of the BHI and SDA Agar Plates

The sterile swab dipped into the inoculum tube was rotated against the side of the tube (above the fluid level) using firm pressure, to remove excess fluid. The dried surface of a BHI agar plate was inoculated by streaking the swab three times over the entire agar surface. Leaving the lid slightly ajar, the plate was allowed to sit at room temperature for 3 to 5 minutes, but no more than 15 minutes, for the surface of the agar plate to dry before proceeding to the next step.

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Preparation of samples

Group		Sub-group
Group-I:	Positive control	A – *Enterococcus faecalis* B – *Candida Albicans*
Group-II:	2% CHX	A – *Enterococcus faecalis* B – *Candida Albicans*
Group-III:	5% SPA	A – *Enterococcus faecalis* B – *Candida Albicans*
Group-IV:	10% SPA	A – *Enterococcus faecalis* B – *Candida Albicans*
Group-V:	5% $CA(OH)_2$	A – *Enterococcus faecalis* B – *Candida Albicans*
Group-VI:	10% $CA(OH)_2$	A – *Enterococcus faecalis* B – *Candida Albicans*

Agar cup bioassay method

All of the reagents extract and culture media were bought to the room temperature. Inoculums were prepared 2-4 hrs prior to starting the experiment. All the petri dishes were labelled as per strains .The standardized (McFarland standard 0.5) inoculum of the test strains were spread evenly on the agar plates. With the help of sterile test tube cups/wells, sizing was 6mm in diameter which can hold 100 microliters of the medicament were punched into the agar. Fixed volumes (100 microliters) of *Spilanthes acmella* flower head extract in concentrations of 5% and 10%, 2% chlorhexidine gel, 5% and 10% of calcium hydroxide were then transferred by micropipettes under aseptic conditions. The plates were maintained at room temperature for 1hr to allow uniform spread of the solvents and then they were incubated at 37° C for up to 24-48 hrs. Mean zone of inhibition for all the test medicaments were measured after 48 hrs by using mm scale and the diameter was noted.

Statiscal Analysis

The zone of inhibition (mm) was compared for *E. faecalis* and *C. albicans* separately between the six groups using one-way analysis of variance (ANOVA) with treatment the factor. Post-hoc Tukey's test was used for pair-wise comparisons. Since data was not normally distributed a non-parametric ANOVA (Kruskal-Wallis test) was also used for comparison of zones of inhibition for the two species by comparison of mean ranks. Median test was also used to compare the groups for zone of inhibition *for E. faecalis* and *C. albicans* separately.

Results

TABLE NO.1: Mean Zone Of Inhibition

		N	Mean	Median	SD	SEM	95% C.I.		Min.	Max.
							Lower	Upper		
Enterococcus fecalis	2% CHX	6	29.7183	29.055	1.71686	.70091	27.9166	31.5201	28.80	33.20
	5% SPA	6	.0000	0.000	.00000	.00000	.0000	.0000	.00	.00
	10% SPA	6	15.4183	15.250	1.73874	.70984	13.5936	17.2430	13.56	17.45
	5% Ca(OH)2	6	10.0500	10.050	.20736	.08466	9.8324	10.2676	9.80	10.30
	10% Ca(OH)2	6	12.9667	12.950	.38816	.15846	12.5593	13.3740	12.50	13.50
	Total	30	13.6307		9.82119	1.79310	9.9634	17.2980	.00	33.20
Candida	2% CHX	6	31.4667	31.950	1.97855	.80774	29.3903	33.5430	28.90	33.50

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albicans	5% SPA	6	.0000	0.000	.00000	.00000	.0000	.0000	.00	.00
	10% SPA	6	18.3983	18.475	.75671	.30893	17.6042	19.1925	17.49	19.30
	5% Ca(OH)2	6	10.1000	10.150	.20976	.08563	9.8799	10.3201	9.80	10.30
	10% Ca(OH)2	6	13.5167	13.500	.38166	.15581	13.1161	13.9172	12.90	14.00
	Total	30	14.6963		10.54396	1.92506	10.7592	18.6335	.00	33.50

TABLE NO.2: One-way analysis of variance (ANOVA)

		Sum of Squares	Df	Mean Square	F	Sig.
Enterococcus fecalis	Between Groups	2766.395	4	691.599	560.953	<0.0001
	Within Groups	30.823	25	1.233		
	Total	2797.218	29			
Candida albicans	Between Groups	3200.694	4	800.173	855.444	<0.0001
	Within Groups	23.385	25	.935		
	Total	3224.079	29			

TABLE NO.3: Post-hoc Tukey's test

Dependent Variable	(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% C.I.	
						Lower	Upper
Enterococcus fecalis	2% CHX	5% SPA	29.71833*	.64107	<0.0001	27.8356	31.6011
		10% SPA	14.30000*	.64107	<0.0001	12.4173	16.1827
		5% Ca(OH)2	19.66833*	.64107	<0.0001	17.7856	21.5511
		10% Ca(OH)2	16.75167*	.64107	<0.0001	14.8689	18.6344
	5% SPA	2% CHX	-29.71833*	.64107	<0.0001	-31.6011	-27.8356
		10% SPA	-15.41833*	.64107	<0.0001	-17.3011	-13.5356
		5% Ca(OH)2	-10.05000*	.64107	<0.0001	-11.9327	-8.1673
		10% Ca(OH)2	-12.96667*	.64107	<0.0001	-14.8494	-11.0839
	10% SPA	2% CHX	-14.30000*	.64107	<0.0001	-16.1827	-12.4173
		5% SPA	15.41833*	.64107	<0.0001	13.5356	17.3011
		5% Ca(OH)2	5.36833*	.64107	<0.0001	3.4856	7.2511
		10% Ca(OH)2	2.45167*	.64107	0.006	.5689	4.3344
	5% Ca(OH)2	2% CHX	-19.66833*	.64107	<0.0001	-21.5511	-17.7856
		5% SPA	10.05000*	.64107	<0.0001	8.1673	11.9327
		10% SPA	-5.36833*	.64107	<0.0001	-7.2511	-3.4856
		10% Ca(OH)2	-2.91667*	.64107	0.001	-4.7994	-1.0339
	10% Ca(OH)2	2% CHX	-16.75167*	.64107	<0.0001	-18.6344	-14.8689
		5% SPA	12.96667*	.64107	<0.0001	11.0839	14.8494
		10% SPA	-2.45167*	.64107	0.006	-4.3344	-.5689
		5% Ca(OH)2	2.91667*	.64107	0.001	1.0339	4.7994

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Candida albicans	2% CHX	5% SPA	31.46667*	.55839	<0.0001	29.8268	33.1066
		10% SPA	13.06833*	.55839	<0.0001	11.4284	14.7082
		5% Ca(OH)2	21.36667*	.55839	<0.0001	19.7268	23.0066
		10% Ca(OH)2	17.95000*	.55839	<0.0001	16.3101	19.5899
	5% SPA	2% CHX	-31.46667*	.55839	<0.0001	-33.1066	-29.8268
		10% SPA	-18.39833*	.55839	<0.0001	-20.0382	-16.7584
		5% Ca(OH)2	-10.10000*	.55839	<0.0001	-11.7399	-8.4601
		10% Ca(OH)2	-13.51667*	.55839	<0.0001	-15.1566	-11.8768
	10% SPA	2% CHX	-13.06833*	.55839	<0.0001	-14.7082	-11.4284
		5% SPA	18.39833*	.55839	<0.0001	16.7584	20.0382
		5% Ca(OH)2	8.29833*	.55839	<0.0001	6.6584	9.9382
		10% Ca(OH)2	4.88167*	.55839	<0.0001	3.2418	6.5216
	5% Ca(OH)2	2% CHX	-21.36667*	.55839	<0.0001	-23.0066	-19.7268
		5% SPA	10.10000*	.55839	<0.0001	8.4601	11.7399
		10% SPA	-8.29833*	.55839	<0.0001	-9.9382	-6.6584
		10% Ca(OH)2	-3.41667*	.55839	<0.0001	-5.0566	-1.7768
	10% Ca(OH)2	2% CHX	-17.95000*	.55839	<0.0001	-19.5899	-16.3101
		5% SPA	13.51667*	.55839	<0.0001	11.8768	15.1566
		10% SPA	-4.88167*	.55839	<0.0001	-6.5216	-3.2418
		5% Ca(OH)2	3.41667*	.55839	<0.0001	1.7768	5.0566

**Sig. at p<0.05*

TABLE NO.4: Median test for E. faecalis

	Group				
	2% CHX	5% SPA	10% SPA	5% Ca(OH)2	10% Ca(OH)2
Enterococcus fecalis > Median	6	0	6	0	3
<= Median	0	6	0	6	3

TABLE NO.5: Kruskal-Wallis test for E. faecalis

	Group	N	Mean Rank
Enterococcus fecalis	2% CHX	6	27.50
	5% SPA	6	3.50
	10% SPA	6	21.50
	5% Ca(OH)2	6	9.50
	10% Ca(OH)2	6	15.50
	Total	30	

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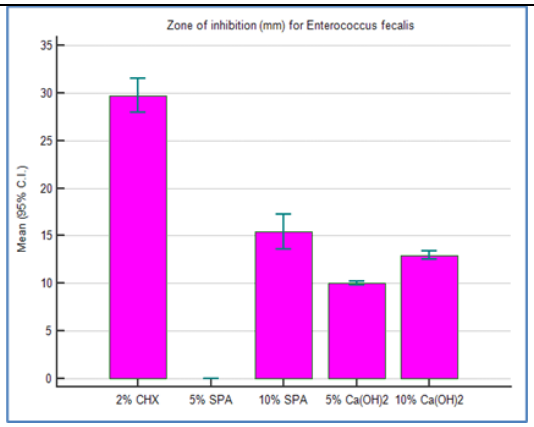
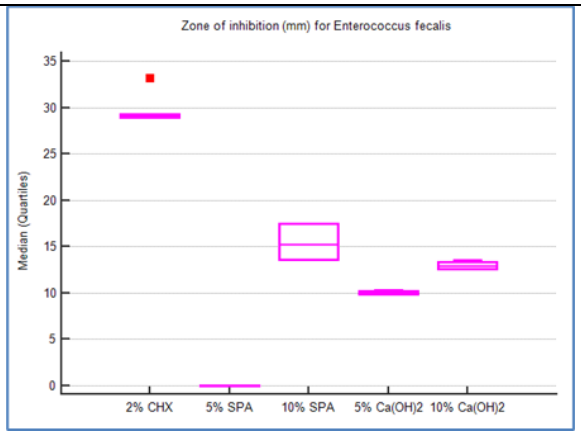


Graph No.1:ZOI for Enterococcus faecalis (Mean)



Graph No.2:ZOI for Enterococcus faecalis (Median)

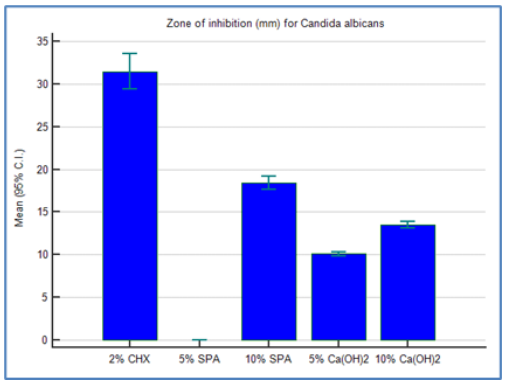
TABLE NO.6: Median test for Candida albicans

Frequencies

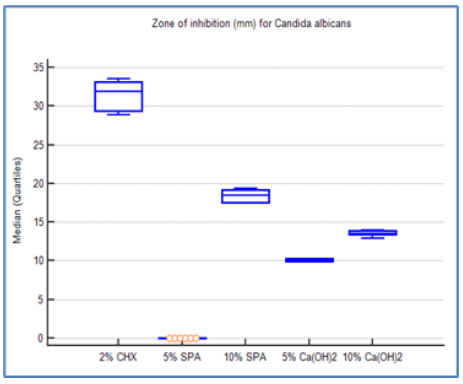
		Group				
		2% CHX	5% SPA	10% SPA	5% Ca(OH)2	10% Ca(OH)2
Candida albicans	> Median	6	0	6	0	3
	<= Median	0	6	0	6	3

TABLE NO.7: Kruskal-Wallis test for Candida albicans

	Group	N	Mean Rank
Candida albicans	2% CHX	6	27.50
	5% SPA	6	3.50
	10% SPA	6	21.50
	5% Ca(OH)2	6	9.50
	10% Ca(OH)2	6	15.50
	Total	30	



GRAPH NO. 3:
ZOI for Candida albicans (Mean)



GRAPH NO.4:
ZOI for Candida albicans (Median)

All analysis were done using PC based program MedCalc Statistical Software version 14.8.1 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2014). All testing was done using two-sided tests at alpha 0.05.

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Discussion

Microorganisms are sufficiently capable of surviving in the dentinal tubules and canal ramifications and are unapproachable to mechanical instrumentation and irrigation. *Enterococcus faecalis* and *Candida albicans* are the most predominant microorganism entailed in persistent root canal infections.[3, 4]In order to accomplish root canal systems devoid of bacteria, it is necessary to use intracanal medicaments.[4]

In the plethora of medicaments, calcium hydroxide has survived the test of time. However, regardless of its broad clinical use, it has some limitations such as the antibacterial activity is fugacious, lacks uninterrupted release, is radiolucent, and has limited action against *E.faecalis* and *C.albicans.* Chlorhexidine is presently the gold standard in intracanal medicament owing to its wide spectrum of bactericidal and antifungal activity.The most distinguishing property of chlorhexidine is its substantivity, which stems from its ability to interact with the dentine and inhibiting dentinal proteases.[5,6,7,8] However, CHX pose few disadvantages such as : a)it is not radiopaque ,b) does not provide physical barrier , c) some studies have reported it to be cytotoxic.[9,10,11]

With the commercial intracanal medicament posing the risk of cytotoxicity and their inability to eradicate bacteria, there is an up rise in employing natural plants extract in dentistry. *Spilanthes acmella* has been regarded with great favor in folklore treatment for toothache, gum and throat infections. It is classified as *'Compositae'* family and genus *'Spilanthes'*. It is broadly spread in India, Sri Lanka, Malaya, America, Australia and Borneo. Its prime constituent being a pungent isobutylamide "spilanthol", which is a known for its bactericidal properties. Also it has immune stimulating alkamide.In-vitro studies have demonstrated potent bactericidal activity against familiar root canal pathogens such as *Enterococcus faecalis, Staphylococcus aureus, Escherichia coli, Streptococcus mutans, Candida albicans, Pseudomonas aeruginosa, Staphylococcus albus, Bacillus subtilis and Kelbsiella pneumoniae*.[12,13,14,15] Thus, in the pursuit of finding an option to commonly used commercial intracanal medicament, extract of flower heads of *Spilanthes acmella* were given a whirl to ascertain its antimicrobial potential against the predominant endodontic pathogens *Enterococcus faeecalis* and *Candida albicans* , when used as a intracanal medicament.

A Soxhlet apparatus invented in the year 1879 by Franz von Soxhlet is a laboratory extractor, primarily intended for the extraction of a lipid from a solid material. Nonetheless, the extraction was not restricted to only lipids. In general, a Soxhlet extractor is solely needed where the sought after compound has finite solubility in a solvent, and the contaminant is indissoluble in that solvent. So, in the present study it was utilized to draw out the flower head extract of *Spilanthes acmella* with petroleum ether as a solvent.[16]

In the present study, agar diffusion bioassay was used for testing the antimicrobial efficacy of different intracanal medicaments as it creates a direct comparison of the medicament against test microorganisms and provides visual indication of the potential to eliminate microorganisms in the local microenvironment of the root canal system. .[17]The advantages of this method are - easy to perform, cost effective, competent to test large numbers of microorganisms and antimicrobial agents, and easy to interpret results provided.Hence, it was the method of choice in the current study.[17]

Spilanthes acmella extract and calcium hydroxide in the concentration of 5% and 10% were opted as it showed eminent antibacterial action in a study performed by Sathyaprasad et al[17]. Likewise, 2% chlorhexidine gel was used as it is established that it has bactericidal effect at this concentration. The results of the present study, depicted 10% *SPA* extract to be superior to both 5% and 10% of calcium hydroxide against *E.faecalis* with significant difference of $P<0.0001$ and $P = 0.006$, respectively. (Table no.-3 and Graph no.-1 and 2).Similary, for *C. albicans*, 10% *SPA* extract exhibited a significant difference of $P<0.0001$ for both 5% and 10% of calcium hydroxide. (Table no.-3 and Graph no. - 3 and 4)

The likely argument for *E.faecalis* surviving in presence of calcium hydroxide stems from its capability of exerting pH homeostasis because of the cytoplasmic buffering activity apart from the capacity of functioning as proton pump. When the negatively charged hydroxyl ions of calcium

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hydroxide infiltrate the bacterial cytoplasm, the pH rises and so the proton pump drive the positively charged ions into the cell to make the cytoplasm acidic, thereby blocking enzymatic inhibition. Even though, *E.faecalis* is incapable of surviving at pH 11.5 or greater, but due to buffering effect of dentine, the pH drops, making it possible for it to survive. Thus, the result conforms to the previous studies.[18, 19, 20, 21] It was also observed that calcium hydroxide did not effectively kill *C.albicans*. The basis might be the fact that *Candida albicans* prefer an alkaline environment to grow. Also the presence of calcium ions facilitates morphogenesis and adhesion of *Candida*. This is in accordance to the study of Sen et al, which states that in presence of yeast infection in root canal; its use may promote yeast to proliferate between the treatment visits. [22, 23,24,25,26]

The outcome of this study has affirmed that *Spilanthes acmella* has noteworthy antibacterial and antifungal activity against *E.faecalis* and *C.albicans*. Nakantani et al suggested that the antimicrobial property of *S. acmella* is due to the presence of flavonoids and tannins. Flavonoids curb the production of free radicals and bacterial virulence factors. Tannins act by divesting the substrate essential for the growth of microorganism, suppressing extracellular microbial enzymes and through the process of inhibiting microbial metabolism by ion privation.[27, 28, 29] The antifungal action of *SPA* is on account of spilanthol and non-volatile sesquiterpenoids. Spilanthol causes enzyme inhibition whereas sesquiterpenoids have cytotoxic property.[30, 31,32,34,35] Nonetheless, 5% of *SPA* extract expressed null value, which is in direct contrast to the study by Sathyaprasad et al. The probable reason the presence of impurities or in the error in making the extract.

In the study, 2% Chlorhexidine gel showed dominance over both *SPA* extract and calcium hydroxide. The superior antibacterial effect on *E.faecalis* is due to its capacity to denature the bacterial cell wall while making pores in their membranes making it vulnerable. Also it inhibits biofilm formation which is one of the essential factors for *E.faecalis* to survive.[33,37,38] (Table no.-1, 3 and 5 and Graph no.1 and 2).The antifungal action of 2% CHX gel is because of its intense inclination of binding to the surrounding tissues which prevent initial attachment and possibly prevent further aggregation and biofilm colonization of yeast. The effective CHX pH is 5.5 – 7 which antagonize the growth of *C.albicans* as they prefer alkaline environment for its growth.[33, 36] (Table no.-1, 3 and 7 and Graph no. - 3 and 4)

The present study has demonstrated striking antibacterial and antifungal effect of *Spilanthes acmella* flower head extract. Hence, the study has fueled the possibility of it being assimilated in the dental science and also to be employed as intracanal medicament against most persistent endodontic pathogens *E.faecalis* and *C.albicans*.

Conclusion

The results of the study elucidated that *Spilanthes acmella* extract has significant antibacterial and antifungal property.10% of *SPA* extract exhibited strikingly superior antibacterial and antifungal action against *E.faecalis* and *C.albicans* as opposed to both 5% and 10% of calcium hydroxide.However, 5% of *SPA* extract failed to show any degree of antibacterial and antifungal action.2% CHX gel surpassed as an antibacterial and antifungal agent when compared to calcium hydroxide and *SPA* extract.

Nonetheless, advance probing and assays are required to illuminate the antibacterial and antifungal efficiency of *Spilanthes acmella* before proposing it as an ideal intracanal medicament.

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