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Research Article

Antimicrobial Efficacy of *Acmella oleracea* L. (*Spilanthes acmella*) Twigs Aqueous and Ethanol Extracts on Tooth Root Canals microorganisms

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ABSTRACT

Medicinal plants are rich sources of phytochemicals and have been recognized to possess a wide range of properties including, antibacterial, anti-inflammatory, and anti-carcinogenic actions. *Acmella oleracea* commonly referred to as *Spilanthes* or "toothache herb". It is flowers and leaves are traditionally chewed or used in the form of a tincture or paste and applied at site of toothache, gum and throat infection hence need to test their antimicrobial activity on root canal cell lines. Twigs of Acmella *oleracea* were collected, air dried and ground into coarse powder. The maceration method of extraction using aqueous and 80 % ethanol was done. The mixtures were gravity filtered and the filtrates pooled and sterile filtered through a Nalgene ® disposable filter unit with 0.45µm pore size filter. The aqueous filtrates were freeze dried while the ethanol filtrates were reduced under vacuum and the yields determined. The tenfold liquid microdilution method using sterile Microtiter plates 96 U-well with lids was undertaken to determine the Minimum Inhibitory Concentrations and Minimum Bactericidal concentrations of the extracts on pure strains of *Enterococcus faecalis (ATCC 29212), Streptococcus mutans (ATCC 25175), Staphylococcus aureus (ATCC 25923), Fusobacterium nucleatum (ATCC 25586), Lactobacillus acidophilus (ATCC 4356) and Candida albicans (ATCC 24433).* Growth was determined as optical density at 630 nm after incubation for 24, and 48hrs at 37^{O}C. Bacterial growth increased especially at 50 mg/ml and 25mg/ml extract concentrations at 24 hours, and 48hours incubation. The mean bacterial inhibition was 25.74% at 95% CI [28.81, 22.67]. Most of tested microorganism showed resistance to these extracts while 2% CHX showed higher growth inhibition.
Conclusion*: Acmella oleracea* L aqueous and ethanal extracts generally potentiated growth of the tested root canal microorganisms. Although, 50mg/ml ethanol extracts showed bacteriostatic growth inhibition against *S. mutans* ATCC® 25175™, *L. acidophilus* ATCC® 4356™ and with *S. aureus* ATCC® 25923™ showing complete resistance.
Keywords*: Acmella oleracea* L., aqueous and ethanol extracts, root canal microorganisms.

INTRODUCTION

Microorganisms are involved in the development of caries which if untreated progress to affect pulp. Dental pulp inflammation (pulpitis) is commonly caused by microorganisms [1] which reach the pulp tissue through; caries, open cavities, periodontal disease, leakage of microbes, or their antigens around lateral canals, or leaking cavity margins of restorations and the blood stream [2]. The microorganisms frequently detected in primary infections, secondary and reinfection in root canals includes *E. faecalis, S. mutans, S. aureus, L. acidophilus, F. nucleutum* and *C. albicans* [3,4,5].

To eliminate these microorganisms an irrigant is required which should have antiseptic effects, be bacteriostatic, bactericidal, and biocompatible with minimal peri-radicular tissue effect. Unfortunately, most of the irrigants, including 5.25 % Sodium hypochlorite (NaOCl), 2% Chlorhexidine gluconate (CHX) and 17 % Ethylene diaminetetraacetic acid (ETDA) do not meet all these requirements [6,7,8]. An alternative irrigant to these conventional agents is needed and medicinal plants analysis as potential source of safe irrigants remains largely uninvestigated.

Acmella oleracea commonly referred to as *Spilanthes* or "toothache herb" belongs to Asteraceae Compositae family [9,10.] The flowers and leaves are chewed, or tincture made, or paste applied at the site of toothache, gum and throat infection. Chewing them is said to have numbing effect to the tongue and gums [9,10]. The leaves are reported to contain important phytoconstituents such as alkamides (spilanthol), triterpenoidal saponins, amino acids and

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alkaloids [11]. The flower heads and roots are said to contain high amounts of isobutyl amide [12, 13].

Extracts containing spilanthol have been used to treat toothache and commercial pastes are indicated for painful mouth and minor mouth ulcers [14, 15]. Also, spilanthol has been incorporated in toothpastes and mouth rinses with the aim of providing a lasting fresh minty flavor.

The aim of the current study was to evaluate the antimicrobial efficacy of twigs aqueous and ethanol extracts of *Acmella oleracea* L on root canal biofilms associated with unfavorable root canal treatment outcome and reinfection.

MATERIALS AND METHODS

Plant material

The twigs of *Acmella oleracea L.* were collected after identification by a taxonomist. They were air dried under shade at room temperature to remove most of the water then ground into a coarse powder and placed in pre-labeled polythene bags.

Extraction procedure

One hundred grams of *Acmella oleracea L.* coarse powder was weighed on Kerne PL2100-2, electronic precision balance (KERN & Sohn GmbH Balingen-Frommern, Germany) and added into a round bottom flask. One litre of sterile distilled water was added, stirred properly, and left to soak for 3days at room temperature. The second and third extraction was done in 500ml and 250ml sterile distilled water, respectively.

At the end of each soaking period, the mixtures were gravity filtration through Whatman No.1 (Whatman Ltd. England) filter paper to remove most of the plant materials. The filtrates were pooled and sterile filtered through Nalgene ® disposable filter unit (Thermo Fisher Scientific, Inc. Waltham, MA USA.) containing 0.45µm pore size filter. Thirty ml of the aqueous filtrates were aliquoted into 50ml Falcón® Centrifuge tubes and frozen at -20 ºC before freeze drying at (Jouan LP-3) -50 ºC and 0.3 mbars vacuum. The mass obtained was weighed and the percentage extract yield determined and stored at 4 º C.

Ethanol extraction protocol was like the aqueous except that, 80% ethanol was used, and the filtrates were reduced and concentrated using rotary evaporator (Heidolph, Laborota 4000, Schwabach, Germany) at 150-200rmp, 80º C under vacuum. The partially solid, sticky wet extracts were placed into 10ml pre weighed, sterile airtight bottles and transferred to Memmert hot air-drying oven (Memmert GmbH & Co. KG, Germany), set at 40º C for drying.

Microorganisms

Pure strains of freeze dried cultures of *Enterococcus faecalis* (*E. faecalis, ATCC*® 29212™), *Streptococcus mutans* (*S. mutans* ATCC® 25175™), *Staphylococcus aureus subsp. aureus* (*S. aureus* ATCC® 25923™), *Fusobacterium nucleatum* (*F. nucleatum* ATCC® 25586™), *Lactobacillus acidophilus* (Moro) Hansen and Mocquot (*L. acidophilus* ATCC® 4356™) and *Candida albicans* (*C. albican* ATCC® 24433™) were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA).

Preparation and storage of inoculum

The stock cultures were maintained below −80°C or as recommended by ATCC for each microorganism. The frozen stocks of *E. faecalis (*ATCC 29212) and *S. mutans* (ATCC 25175), were grown in Brain Heart Infusion broth (BHI Oxoid) under 95% N_2 and 5%CO_2 (v/v), *F. nucleatum (*ATCC 25586) in Modified chopped meat medium in an anaerobic chamber (80% N_2, 10% CO_2 and 10% H_2), *S. aureus* strains (ATCC 25923) in Trypticase Soy, *L. acidophilus* (ATCC 4356), in Lactobacilli MRS broth (Oxoid) under 95% N_2 and 5% CO_2 (v/v) and *C. albican* (ATCC *24433)* in Sabouraud agar in 5% carbon dioxide. The entire microorganisms were incubated at 37^{O}C and for each organism (primary subculture) and a second subculture (day 1 working culture) was made.

Microbial growth was determined visually by changes in turbidity compared to 0.5 McFarland Standards (1.5 X 10^8 CFU/mL) at 24 hours for the bacteria and 48hrs for *C. albican*. Strict observances of experimental protocols were undertaken to ensure purity of microorganisms.

Minimal Inhibitory Concentration

Ten mg of the freeze-dried *Acmella oleracea* L. aqueous extract was resuspended in 2 mL of sterile distilled water and another 10mg of ethanol extract resuspended in the 20 % (v/v) DMSO to make 50 % stock solutions.

Sterile Microtiter plates 96U well with lids (8 by 12 matrix, Bioster.S.P. A, Italy), with retention capacity of more than 200 µl per well were used. 160 µl of sterile growth media was drawn with adjustable 20-200 µl micropipette (Eppendorffs® Research® Plus Hamburg, Germany) using 20 µl sterile disposable micropipette tips into the wells. Tenfold microdilution (Clinical and Laboratory Standards Institute (CLSI) 2008, 20011)[16] with modification was used. Twenty µl of 50 mg/ml extract solution was drawn and added to the first well, mixed properly and 20 µl solution drawn and added to the second well. This was repeated for all the wells and last 20 µl from the 10th well was discarded.

Each well was inoculated with 10 µl of bacterial suspension at a density of 1.5x10^8 CFU/mL using 0.5-10 µl Gilson adjustable micropipette (Gilson Inc. Middleton, WI. USA). The 11th well contained broth and microorganisms and 12th broth plus extract for intra-experimental control. Baselines readings were obtained at 1 hour before incubation in Heratherm™ Compact Microbiological Incubators at 37°C for 24 and 48 hrs. Growth of microorganisms was read of as optical density using BioTek Elisa Photometer (BioTek Instruments, Inc. USA) at 630 nm. Sterile distilled water and 2% CHX were used as negative and positive control, respectively. All microorganisms were tested independently, each carried out in triplicate compared with the efficacy of 5.25% NaOCl and 17% EDTA liquid.

Minimum Inhibitory Concentrations were calculated based on the density of growth control and expressed as the lowest extract concentration that resulted in 80% growth reduction, while Minimum Bactericidal Concentrations was the lowest concentration of the plant extracts that did not yield any bacterial growth upon subculture compared to that of the extract free growth control.

RESULTS

The extracts yield from100gms powder were 5.90gm (0.059%) and 5.66gms (.0566%) for aqueous and ethanol extracts, respectively.

One thousand one hundred and forty (1140) samples of *Acmella oleracea* extracts were investigated against six root canal microorganisms. The aqueous and ethanol extracts had minimal growth inhibition on the selected root canal microorganisms. Bacterial growth increased especially at 50 mg/ml and 25 mg/ml extract concentrations at 24 hours and 48hours. The mean bacterial inhibition was 25.74% at 95% CI [28.81, 22.67]. The median of inhibition was similar for the aqueous and ethanol extracts (Figure 1).

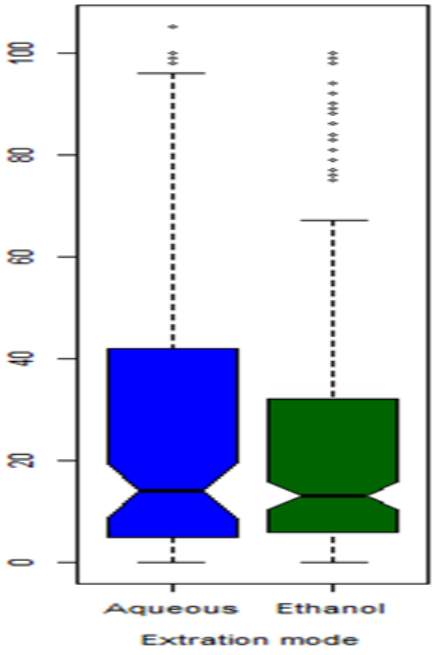

Figure 1: Distribution of mean growth inhibition of *Acmella oleracea* extracts against the six root canal microorganisms. Further these extracts resulted in either normal or increased growth against six microorganisms at 24 hours and 48 hours incubation (Table 1).

Lactobacillus acidophilus, *S. mutans* and *C. albicans* were the most resistant to aqueous extracts in that order while S. *mutans* and *S. aureus* were the most resistant to 80% ethanol extracts at 24 hours, and 48 hours incubation time, respectively. The aqueous extracts had no growth inhibition on *E. faecalis* ATCC® 29212) at 24 hours and 48 hours while the ethanol extracts showed very minimal effects with highest inhibition being 23.87 % ± 16.31 achieved with 50 mg/ml concentration at 48 hours. These effects were like those attained with 5.25% NaOCL 28.83% ± 9.60. The 50 mg/ml ethanol extracts concentration attained 58.42% ± 28.66 and 51.42% ± 35.90 with growth inhibition against *Streptococcus mutans* (ATCC® 25175™) cultures at 24 hours and 48 hours, respectively. However, *Staphylococcus aureus* (ATCC® 25923™) cultures growth generally increased especially at 6.25 mg/ml to 50 mg/ml extracts concentrations compared to the growth obtained with growth control (Figure 2).

The ethanol extracts had significant inhibition against *L. acidophilus* Hansen and Mocquot (ATCC® 4356) that reduced with longer incubation as shown in Figure 3. The minimum inhibitory concentration was 25 mg/ml at 24 hours incubation period.

Candida albicans generally showed similar response at 24 hours and 48 hours incubation as those of *F. nucleatum* (Figure 4).

The 50 mg/ml ethanol extract concentration showed inhibitory effects of 49% ± 12.33 against F. *nucleatum* at 48 hours while *C. albicans* had 42.36% ± 1.95, at 24 hours incubation. The inhibition decreased with incubation time for *C. albicans*, although there was variance at $F(1, 19) = 2.1625$, at $p=0.1587$ (One-way ANOVA) the mean inhibition difference was non-significant at $p<0.01$(Tukey's HSD test).

The *Acmella oleracea* aqueous and ethanol extracts growth inhibitions were compared with those of conventional root canal irrigating solutions, where 2% CHX showed higher growth inhibition (Figure 5).

DISCUSSION

Acmella oleracea commonly referred to as *spilanthes* as well as toothache herb because its use traditionally for management of toothache [11-18]. The aqueous extracts of *Acmella oleracea* potentiated growth of the tested microorganisms with highest effects being on *L. acidophilus* followed by *S. aureus*, and *C. albicans*. The enhanced survival of these organisms may be associated with presence of sugars in the crude extracts of this plant especially glucose which has been shown to have a protective effect on *Lactobacillus acidophilus* [19]. These researchers showed that the "survival of *L. acidophilus* increased by more than 1 $\log_{10}$ cycle, even at very low concentrations of maltose and glucose ".

Further the enhanced survival and hence growth has been associated with attainment of critical pH which could have been achieved with these extracts facilitating glycolysis [20]. Although the sugars are part phytochemical present in aqueous extracts [21-23] it was beyond the scope of this study to analyze the amounts, and pH values that may have had an effect and this opens a gap for further research. It could also be that phytoconstituents alkamides (Spilanthol), triterpenoidal, saponins, amino acids and alkaloids [11] were inadequate in aqueous extracts.

The results of this study showed minimal inhibitory effects of *Acmella oleracea* ethanol on *Enterococcus faecalis* with *Staphylococcus aureus* showing complete resistance to all extract concentrations at 24 hour, and 48 hours incubation. Thompson *et al.*, (2012) [24] using agar well diffusion method reported some efficacy with ethanol extract (9mm zone of inhibition) on *L. acidophilus* while the current study found on average 53.8% ± 4.33 growth inhibition at 24 hours, and 48 hours incubation time points. Thedifferences in the investigative methods between the current study and that of Thompson *et al*., (2012) [24], it difficult to draw a direct comparison.

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Table 1: Mean percent growth of root canal microorganisms exposed to *Acmella oleracea* extracts.

Organisms	Time in Hours	Aqueous				80% Ethanol			
				95% CI				95% CI	
		Mean	±SD	Min	Max	Mean	±SD	Min	Max
E. faecalis	24	119.43	22.03	112.46	126.40	86.85	1.80	85.05	88.65
	48	107.88	31.77	97.83	117.92	91.73	2.04	89.70	93.77
S. mutans	24	134.02	40.22	121.30	146.74	134.58	24.57	126.81	142.35
	48	139.98	26.92	131.47	148.49	108.01	7.17	105.74	110.28
S. aureus	24	109.60	17.56	104.05	115.15	107.30	15.78	102.31	112.29
	48	108.00	13.48	103.75	112.29	116.40	18.60	110.52	122.28
L. acidophilus	24	138.70	92.47	109.46	167.94	43.16	31.49	33.36	52.96
	48	152.20	74.48	128.65	175.75	103.96	47.82	88.84,	119.08
F. Nucleatum	48	96.37	35.73	85.07	107.67	87.54	32.84	77.42	97.66
	7*	109.27	35.53	98.03	120.51	104.28	33.47	93.70	114.86
C. albicans	24	114.77	6.48	136.65	140.75	75.61	14.52	71.02	80.2
	48	125.27	18.00	146.51	157.89	83.96	9.65	80.91	87.01

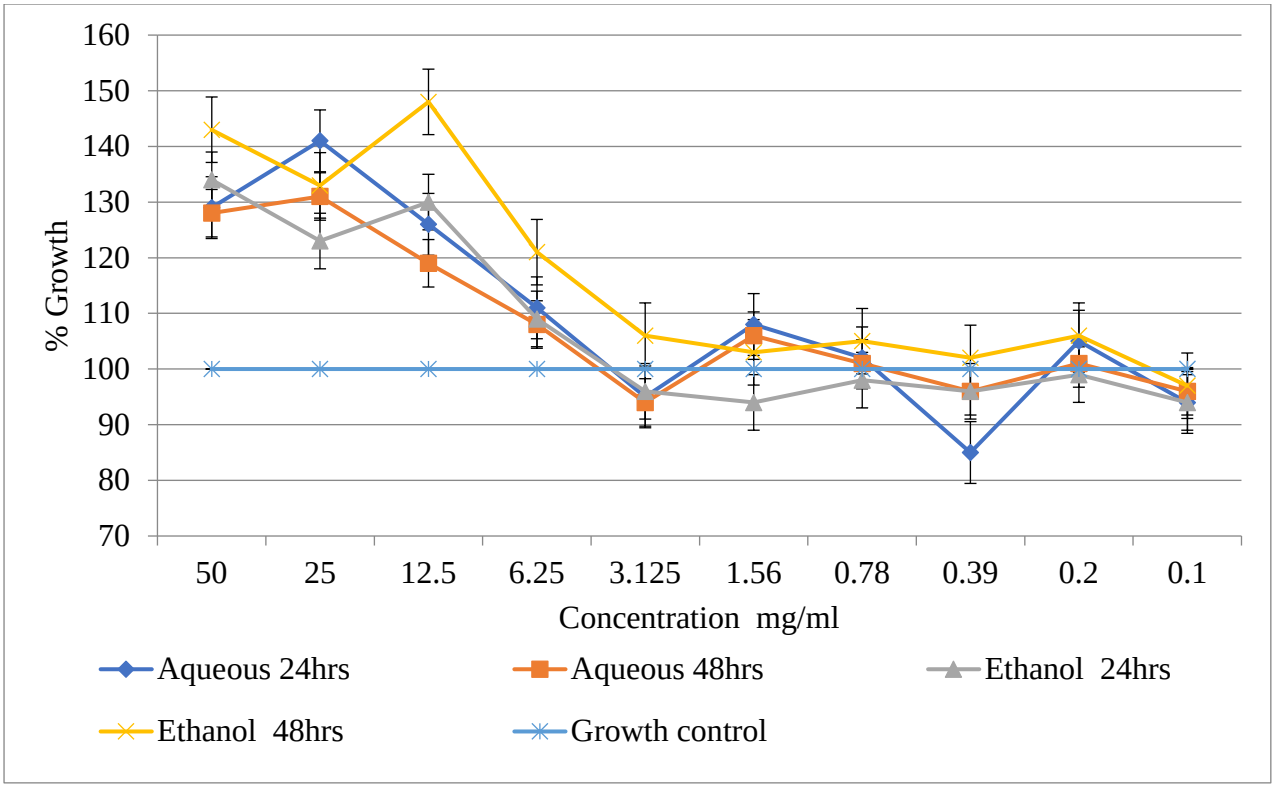

Figure 1: Growth effects of *Acmella oleracea* extracts against *Staphylococcus aureus*.

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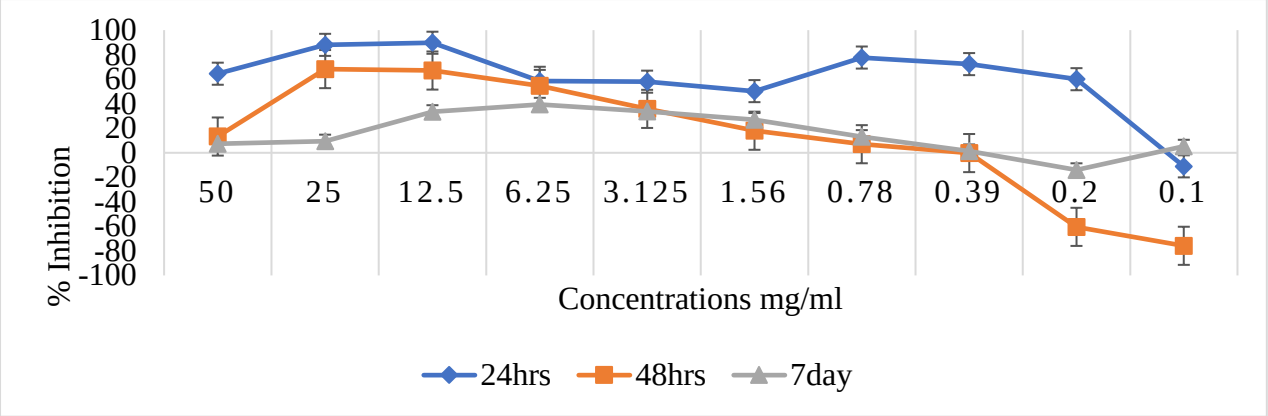

Figure 3: Growth inhibition of *Acmella oleracea* ethanol extracts against *Lactobacillus acidophilus.*

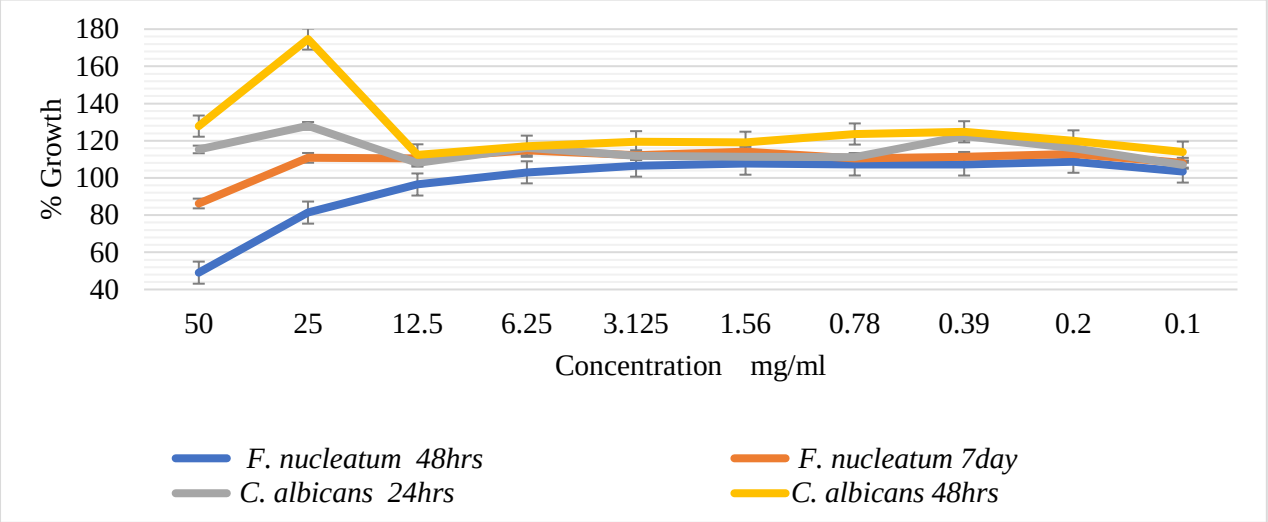

Figure 4: Growth effects of *Acmella oleracea* aqueous extracts against *Fusobacterium nucleatum* and *Candida albicans.*

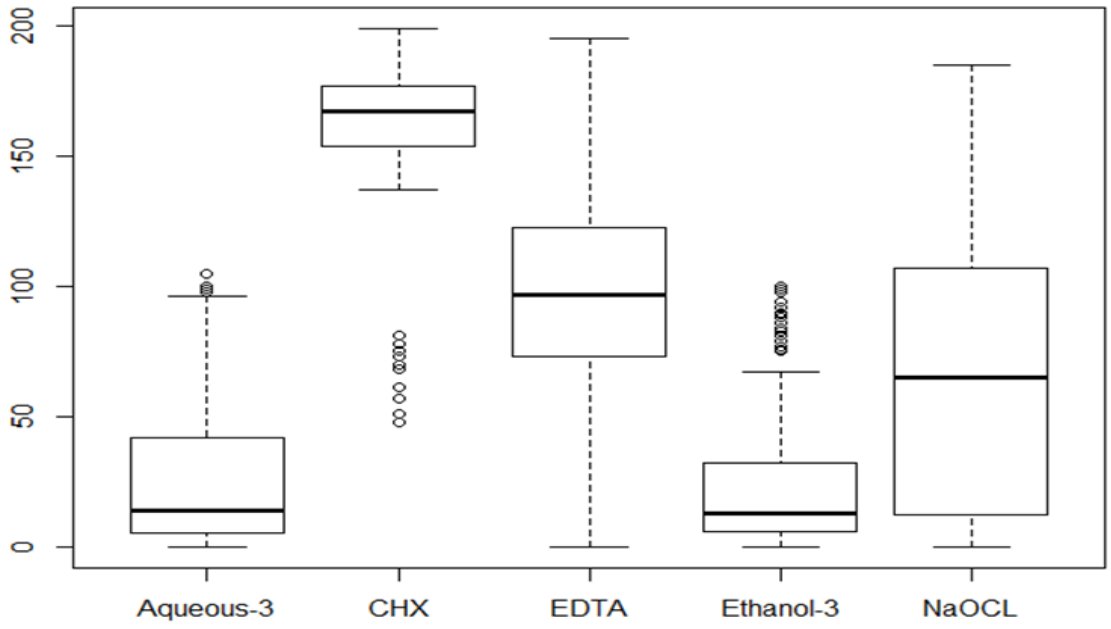

Figure 5: Distribution of mean growth inhibition of *Acmella oleracea* (3) extracts compared with conventional root canal irrigants against root canal microorganisms.

On the other hand, the same researchers[24] found no inhibitory effects with aqueous extracts, which is like the findings of this study despite using higher amounts and concentration 50 µl of a 100 mg/ml extract concentration while current study used 20 µl of 50 mg/ml. The ethanol extract had some growth inhibition on *L acidophilus, S. mutans, and F. nucleatum* and at 24 hours which decreased at 48 hours, and 7 days incubation. *L. acidophilus* growth inhibition decrease with time was statistically significant at $p<0.05$ (Tukey's HSD). This proved the bacteriostatic effects of these extracts.

Spilanthol is the main constituent isolated from many parts of [11-15 17]. It is associated with the analgesic effects achieved in managing toothache by decreasing prostaglandins that affects pain perception [11, 25, 26]. This was tested and demonstrated by Chakraborty *et al*., (2004, 2010) [25, 26] in an experimental animal model. Furthermore, Thompson *et al*., (2012) [24] found no flavonoids and tannin in aqueous extract of *S. acmella* which are associated with antimicrobial activity [21, 22]. This may explain its low antimicrobial efficacy that was found is this study. Also, the few studies that have reported some antimicrobial activity of this plant used dried flowers or leave or stem [11-15, 17, 27] while current study used stem/twigs. The use of different plant parts may have yielded different quantities of the bioactive compounds which have been demonstrated [14,15, 27].

CONCLUSION

The ethanol extract had bacteriostatic efficacy on *Streptococcus mutans* (*S. mutans* ATCC® 25175™), *Lactobacillus acidophilus* (Moro) Hansen and Mocquot (*L. acidophilus* ATCC® 4356™) and *Fusobacterium nucleatum* (*F. nucleatum* ATCC® 25586). While *Staphylococcus aureus subsp. aureus* (*S. aureus* ATCC® 25923™) showed complete resistance.

RECOMMENDATION

Further research is needed to test higher concentrations of purified *Acmella oleracea* ethanol extracts against root canal microorganisms.

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