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Original Article

Comparative Evaluation of Anti Microbial Efficacy of Phytomedicinal Extracts with Chemical Root Canal Irrigants against Enterococcus Faecalis: An in Vitro Study

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ABSTRACT

Introduction: One of the most important objectives of root canal treatment is elimination of microorganisms from the root canal system that is achievable by augmenting mechanical preparation with antimicrobial irrigants. Enterococcus faecalis considered as the main culprit, is the primary organism detected in persistent asymptomatic infections. A myriad of chemical irrigants like sodium hypochlorite and QMix have been used widely yet the quest for ideal root canal irrigant still continues. The global scenario is now showing a trend towards the use of nontoxic plant products. Herbal products have shown a promising role as root canal irrigants. Thus, this in-vitro study intends to identify the phytoactive compounds present in the phytomedicinal extracts and to evaluate and compare the antimicrobial efficacy of the phytomedicinal extracts with chemical root canal irrigants against E. faecalis.

Aim: The aim of this in vitro study is to assess and compare the antimicrobial efficacy of phytomedicinal plant extracts (Neem, Miswak, Cinnamon, Apple cider vinegar) with Sodium hypochlorite and QMix as root canal irrigants against E. faecalis using agar well diffusion assay.

Methodology: The methodology was divided into two phases, first being the identification of phytoactive compounds present in the phytomedicinal extracts by gas chromatography mass spectrometry analysis and the second phase included the evaluation of antimicrobial senstivity of the test agents against E. faecalis, which was performed using agar well diffusion assay.

Results: All phytomedicinal extracts showed significant antimicrobial activity against E. faecalis($p < 0.001$).Cinnamon showed highest antimicrobial activityi.e. highest mean zone of inhibition among all the tested irrigants followed by miswak, QMix, 3% sodium hypochlorite, apple cider vinegar and neem.

Conclusion: Hence, phytomedicinal irrigants have huge potential to be used as root canal irrigant as an alternative to chemical root canal irrigants.

Key Words: *Phytomedicinal extracts, herbal root canal irrigants, herbendodontics, root canal irrigation, agar well diffusion, gas chromatography mass spectrometry analysis, E. faecalis*

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INTRODUCTION

Endodontic infections are known to be polymicrobial in nature, with complex bacterial interactions and a preponderance towards anaerobic species in the root canals of teeth [1]. *Enterococcus faecalis* has gained attention in the endodontic literature, as it has been found to be the main culprit in cases of failed root canal treatment [2, 3] Studies have shown its occurrence in root-filled teeth with the prevalence ranging from 24 to 77% [4]. The inherent antimicrobial resistance and the ability to adapt to a changing environment help *E. faecalis* to persist in the root canals. Effective disinfection of the root canals can be achieved by augmenting mechanical preparation with antimicrobial irrigants. Various irrigating agents have been employed in clinical practice, depending upon their antimicrobial efficacy, cleaning efficiency, lubrication, and biological compatibility. Sodium hypochlorite, though considered as gold standard root canal irrigant, it's use at required concentration is avoided in children due to unpleasant taste, toxicity, and inability to remove the inorganic portion of smear layer. QMix 2 in 1 solution contains a mixture of a bisbiguanide antimicrobial agent (2% chlorhexidine), a poly amino carboxylic acid calcium-chelating agent (17% EDTA), and surfactant. This solution has also demonstrated substantial smear layer removal and antimicrobial properties. In spite of significant development in the field of dentistry, there is no ideal root canal irrigant. Hence, the quest for an alternative irrigant still continues. The

global scenario is now showing a trend towards the use of nontoxic plant products that have been used for thousands of years. In dentistry, phyto medicines have been used as anti-inflammatory, antibiotic, analgesic and sedative agents [5]. Studies have shown that herbal products have a promising role as root canal irrigants. The major advantages of herbal irrigants are safety, easy availability, increased shelf-life, cost-effective, etc [6]. Currently, very few natural products have been identified that might be used as an alternative. Hence, this in-vitro study intends to identify the phytoactive compounds present in the phytomedicinal extracts and to assess and compare the antimicrobial efficacy of the phytomedicinal extracts with chemical root canal irrigants against *E. Faecalis*.

AIM

The aim of this in vitro study is to assess and compare the antimicrobial efficacy of phytomedicinal plant extracts (Neem, Miswak, Cinnamon, Apple cider vinegar) with Sodium hypochlorite and QMix as root canal irrigants against *E. faecalis*using agar well diffusion assay.

MATERIALS AND METHOD

The present in vitro study was conducted in the Department of Pedodontics and Preventive Dentistry, IGGDC, Jammu, in association with Infectious Diseases Division, Council of Scientific & Industrial Research, Indian Institute of Integrative Medicine, Canal Road, Jammu. Ethical approval was taken from institutional ethical committee, IGGDC, Jammu.

PHYTOMEDICINAL EXTRACTS

PREPARATION OF HERBAL EXTRACTS

In this study, the medicinal plants used for the experimental purpose were [Fig.1]:

1) **NEEM (*Azadirachta indica)* -** Mature fresh neem leaves were collected from the medicinal garden of CSIR- IIIM, Jammu.
2) **CINNAMON *(Cinnamomum zeylanicum*)** - Cinnamon barks were purchased from the local market.
3) **MISWAK *(Salvadora persica*)** - Miswak sticks were purchased from the local market.

The taxonomical identification of the plant material was done. The neem leaves, cinnamon bark, and miswak sticks were washed with sterilized distilled water and air-dried at room temperature. The plant material was then pulverized with the help of electric grinder and was sieved to obtain fine powder. Absolute ethanol, ethyl acetate, and butanol were used as solvents for the preparation of plant extracts.

25 gm of neem, cinnamon, and miswak powder was macerated in a conical flask with 200 mL of absolute ethanol, butanol, and ethyl acetate each. The macerated mixture was subjected to continuous shaking for 1 h at a speed of 120 rpm at 37°C. The extract was filtered with Whatman No.1 filter paper for coarse residue. The extraction process was repeated using coarse residue in 200 mL of ethanol, butanol, and ethyl acetate each until exhaustion. The extracts were pooled and filtered through filter paper. The solvent part was removed from the extract by rota evaporation until the extract was completely dry to obtain the crude extract. The extracts were stored in air tight glass vials and maintained at 4°C until required [7]. [Fig.2]For preparation of test solutions, crude plant extracts were dissolved in DMSO. [Fig.3]

4) **APPLE CIDER VINEGAR -** Commercially available apple cider vinegar without any dilution (100%) was used.

Fig 1: Plant Material – (A) Neem Leaves, (B) Miswak Sticks, (C) Cinnamon Bark, (D) Apple Cider Vinegar

Fig 2: Preperation of Herbal Extracts

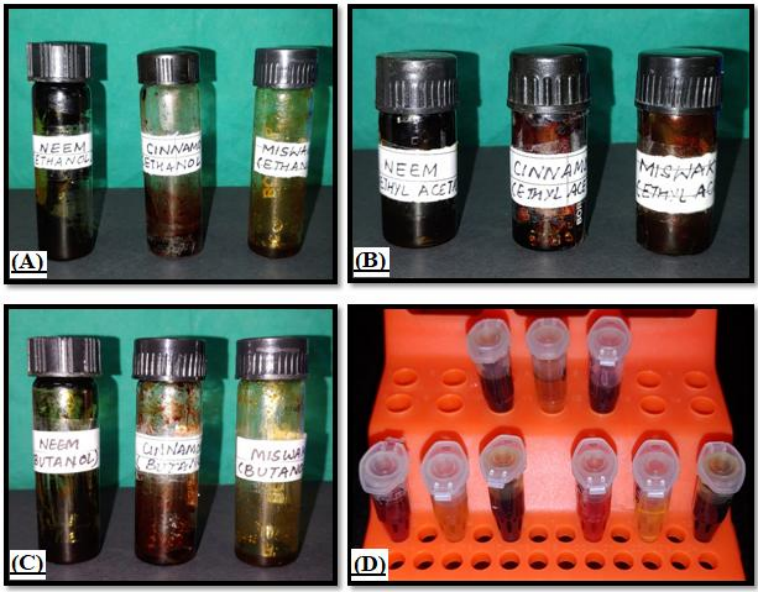


Fig 3: Dried Extracts of Neem, Cinnamon and Miswak (A) Ethanol (B) Ethyl Acetate (C) Butanol. (D) Extracts Dissolved in Dmso

CHEMICAL ROOT CANAL IRRIGANTS

The chemical root canal irrigants used in the study were 3% Sodium hypochlorite and QMix. Normal saline was used as negative control. [Fig.4]

Fig 4: Chemical Rootcanal Irrigants–(A) 3% Sodium Hypochlorite, (B) QmixChemical Rootcanal Irrigants

PREPERATION OF CULTURE MEDIA

Mueller Hinton agar media and Brain Heart Infusion broth were prepared by suspending 3.8 g of Mueller Hinton agar powder and 3.7g of Brain Heart Infusion broth powder respectively in 100 mL of distilled water according to manufacturer's instruction followed by mixing so as to dissolve it completely. The media were further sterilized by autoclaving at 121°C for 15 minutes.

SELECTION AND PREPARATION OF BACTERIAL MICRO-ORGANISM

Microbial strain used in the study is *Enterococcus faecalis* (Microbial Type Culture Collection and Gene Bank, Chandigarh, India; MTCC 2729). A loop full of culture grown on Mueller Hinton agar was inoculated in sterile brain heart infusion (BHI) broth and adjusted spectrophotometrically at 600 nm to an optical density corresponding to match the turbidity of 0.5 McFarland scale. [Fig.5]all the steps were carried out using sterile materials and instruments in Laminar Air Flow, the working surface of which was swabbed with spirit and UV lamp was switched on for 30 min before procedure.

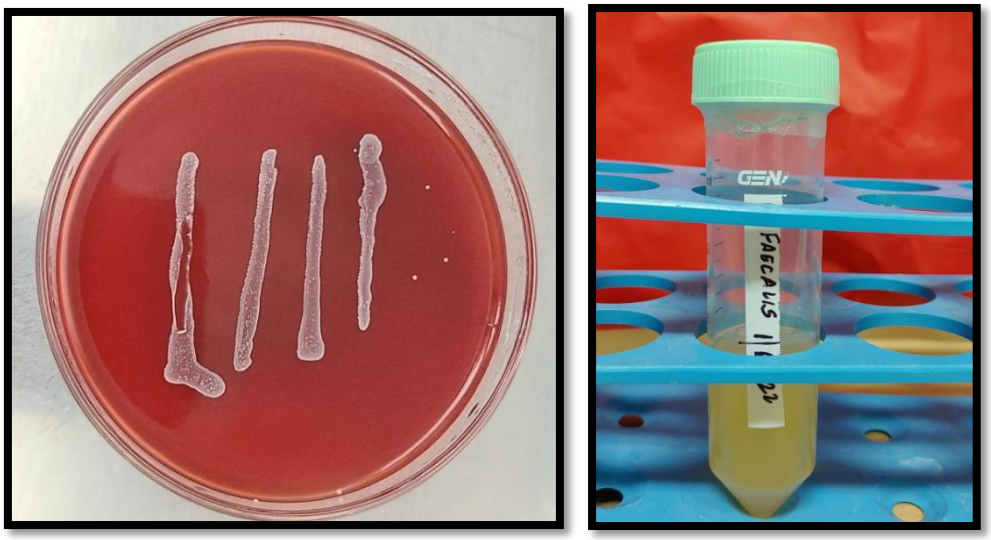


Fig 5: (A) *E. Faecalis* Grown on Agar Plate. (B) *E. Faecalis* Culture in Bhi BrothA. Gas Chromatography Mass Spectrometry Analysis - Identification of Phytoactve Compounds

The herbal extracts were analyzed by Gas Chromatography- Mass Spectrometry (GC–MS) analysis after derivatization. For derivatization, 80 µl of methoxyaminehydrochloride in pyridine (20 mg ml−1) was added to each tube and incubated at 37 °C for 2 h. N-methyl-N-(trimethylsilyl)- trifluoroacetamide (200 µl) was further added and incubated at 37 °C for 1 h. The mixture was transferred to the suitable GC vials with inserts and analyzed for GC–MS.[Fig. 6] The chromatographic analysis were carried out with a Trace GC ultra-gas chromatograph, coupled with a Dual Stage Quadrupole (DSQ) mass spectrometer. A Tr-5 capillary column (30 m×0.25mm i.d.) coated with 0.25µm film 5% phenyl– 95% dimethylpolysiloxane was used for separation. The GC injector was maintained at 250°C, the GC transfer line at 250 °C, and the ion source at 220 °C. The column temperature started with 70 °C hold for 1min, then increased the temperature at the rate of 6 °C/min to 280 °C hold for 3min. Manual injection (1µl) in a purged split mode (1:100) was

conducted and high purity helium was used as carrier gas at a 1ml/min flow rate. The scan range was 35–550m/z and the scan rate was 1000 amu/s. The volume injected was 1µl of 1% solution. The identification of the components was based on a comparison of their mass spectra with those of a computer library (NIST17R.lib MS Search library). Further confirmation was done by referring to retention index (RI) data and comparison with literature data [8].

Fig 6*:*Samples in Gc Vials for Gc-Ms Analysis

B. ANTIMICROBIAL SUSCEPTIBILITY TEST

Agar Well Diffusion assay, a standardized testing method recommended by Clinical and Laboratory Standards Institute (CLSI) [9], was performed to determine the antibacterial activity of phytomedicinal extracts i.e. neem, miswak, cinnamon, apple cider vinegar and chemical root canal irrigants against *E. faecalis*.

AGAR WELL DIFFUSION ASSAY

Well diffusion test is a practical and convenient method for testing multiple antibacterial agents against bacterial strain. Using the well diffusion susceptibility test, antibacterial activity can be detected by challenging bacterial isolates with antibiotic test solution that are placed on the surface of an agar plate that has been seeded with a lawn of bacteria.

Agar well diffusion assay was performed at various concentrations. Petri dishes of 90 mm diameter were used. In each petri dish, Mueller-Hinton agar was poured and swirled to distribute the medium homogenously. After solidification of the agar, wells of 6 mm diameter and 4 mm depth were bored aseptically using pipette tip into the medium. 100uL freshly prepared inoculum containing the bacterial suspension in BHI broth, was spread over the surface of the Mueller-Hinton agar plates with the help of spreader. Using pipettes, the wells were filled with 50 µL of each herbal extract and controls in their respective wells. The plates were left for 30 min at room temperature to allow diffusion of the plant extracts through the agar, and then they were incubated at 37°C for 24 h. After incubation, the plates were observed for zones of inhibition of the microbial growth around the wells. The diameters of these zones were measured in millimeters with a transparent ruler and recorded. The antimicrobial assay was performed in triplicates [10].

STATISTICAL ANALYSIS

Statistical analysis was done by using IBM SPSS VERSION 21. Continuous data were expressed in terms of mean and standard deviation and was compared by using student t test for independent samples and ANOVA test. Qualitative data were expressed in terms of proportion and percentages and compared by using chi square test and fisher exact test. The p-value < 0.05 was considered as statistically significant and other wise non-significant.

RESULTS AND OBSERVATIONS

A. Gas Chromatography Mass Spectrometry Analysis-Identification of Phytoactve Compounds

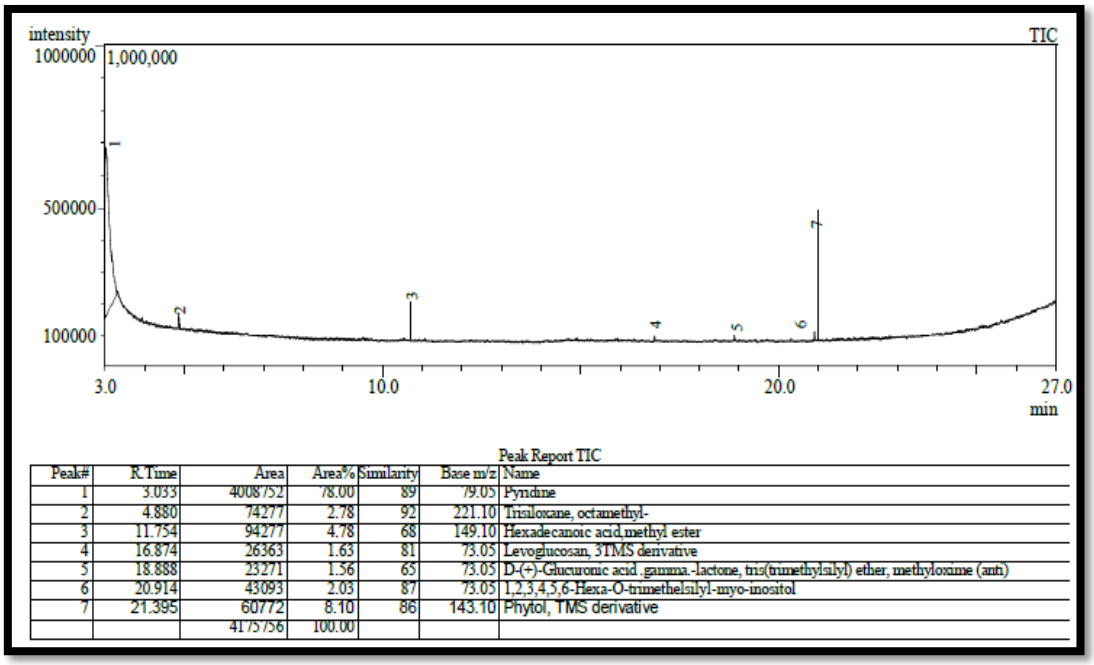
Results of gas chromatography- mass spectrometry analysis of ethanolic extract of neem identified various phytomedicinal compounds such as hexadecanoic acid, levoglucosan, D-(+)-glucuronicacid-gamma-lactone, 1,2,3,4,5,6-hexa-O-trimethelsilyl-myo-inositol, phytol etc. The butanolic extract of neem showed the presence 9-octadecenoic acid-methyl ester, hexadecanoic acid, tridecane-6-methyl-ester, 2-Decanone o-methyloxime, thymol, beta-d-glucopyranoside etc. The ethyl acetate extract of neem shows the presence of tridecane-6-methyl-ester, 1,2,3,4,5,6-hexa-O-trimethelsilyl-myo-inositol, phytol, ethylalpha-D-glucopyranoside etc. [Fig.7(A), (B),(C)]

Results of gas chromatography- mass spectrometry analysis of ethanol extract of miswak shows the presence of various phytocomponents benzyl isothiocyanate, glucuronicacid, etc. The butanolic extract of miswak showed presence of benzaldehyde, n-benzyloctadecenamide, n-hexadecanoic acid, etc. The ethyl acetate extract of miswak showed presence of phytomedicinal compounds such as benzoic acid, benzyl isocyanate, benzaldehyde, benzyl nitrile, thymol,etc.[Fig.8 (A), (B), (C)]

Results of gas chromatography- mass spectrometry analysis of ethanolic extract of cinnamon shows the presence of various phytocomponents (E)-cinnamaldehyde, benzaldehyde, copaene, aniline-2-(3-methoxy-1-propynyl), 1-isopropyl-4,7-dimethyl hexahydronaphthalene, etc. The butanolic extract of cinnamon showed presence of mainly benzaldehyde

dimethyl ester, (E)- cinnamyl acetate, etc.The ethyl acetate extract of cinnamon showed presence of (E)-cinnamaldehyde, benzaldehyde, copaene, naphthalene, benzoic acid-methyl ester, etc. [Fig.9(A), (B),(C)]

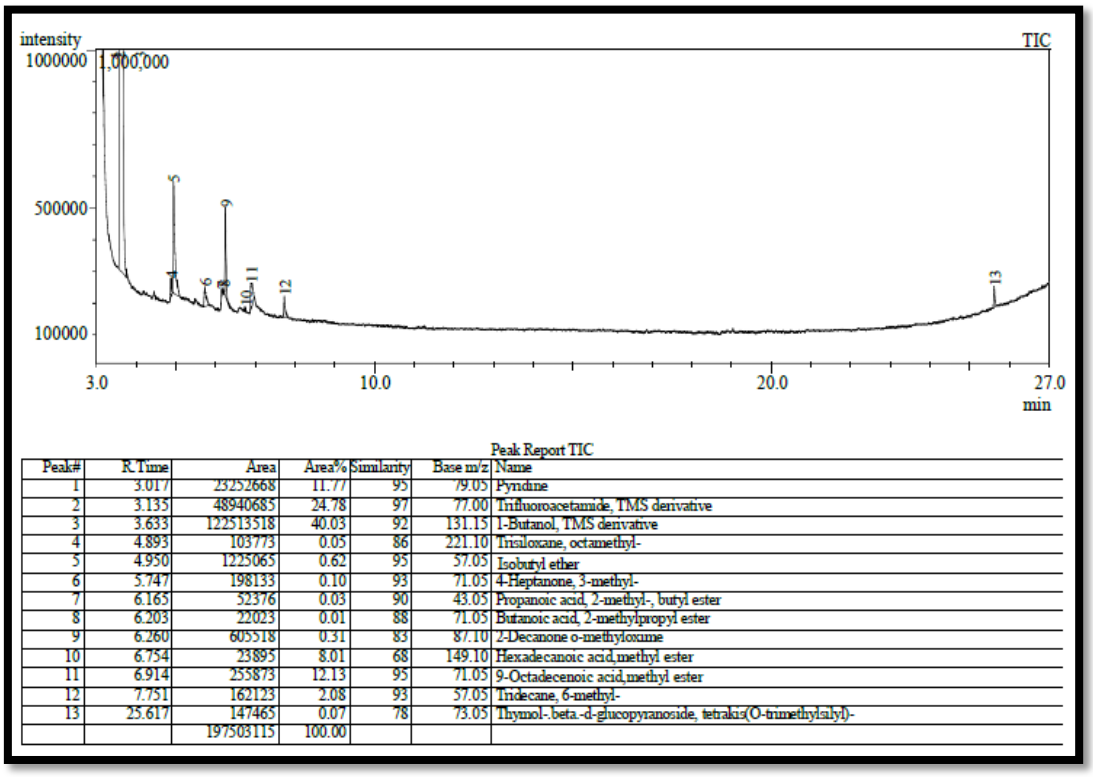
Results of gas chromatography- mass spectrometry analysis of apple cider vinegar shows the presence of various phytoactive components like 1,3-dioxolane-2-acetic acid, 3,3-dimethoxy-2-butanone, butanoic acid-2-methyl-pentyl ester, 3,3-dimethyl-1,2-butanediol, 2,2-dimethoxybutane, 1,3-dioxolane-4-methanol, etc.[Fig. 10]



Peak Report TIC

Peak#	R.Time	Area	Area%	Similarity	Base m/z	Name
1	3.033	4008752	78.00	89	79.05	Pyridine
2	4.880	74277	2.78	92	221.10	Trisiloxane, octamethyl-
3	11.754	94277	4.78	68	149.10	Hexadecanoic acid,methyl ester
4	16.874	26363	1.63	81	73.05	Levoglucosan, 3TMS derivative
5	18.888	23271	1.56	65	73.05	D-(+)-Glucuronic acid .gamma.-lactone, tris(trimethylsilyl) ether, methyloxime (anti)
6	20.914	43093	2.03	87	73.05	1,2,3,4,5,6-Hexa-O-trimethelsilyl-myo-inositol
7	21.395	60772	8.10	86	143.10	Phytol, TMS derivative
		4175756	100.00			

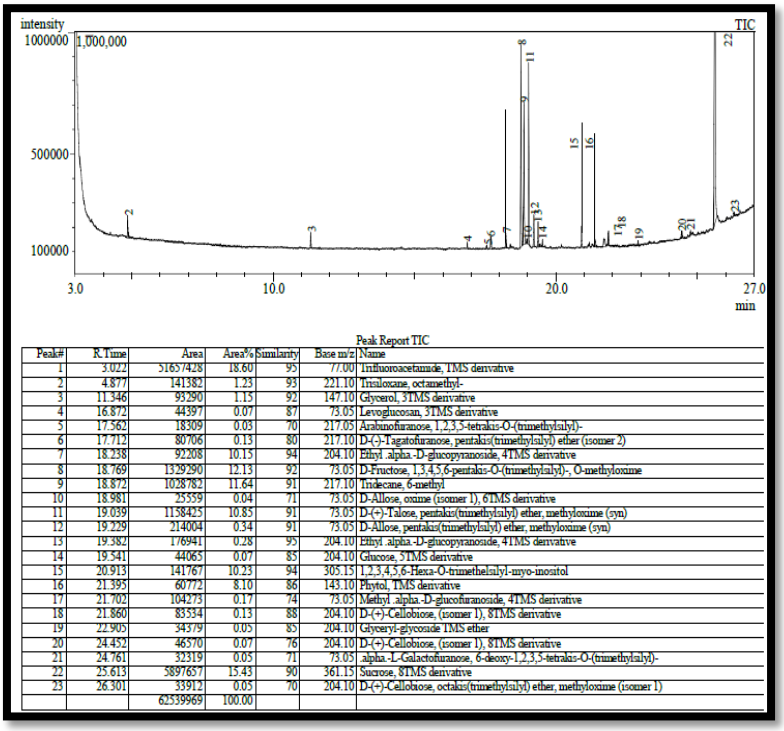
Fig 7(A): Gc-Ms Analysis of Ethanolic Extract of Neem



Peak Report TIC

Peak#	R.Time	Area	Area%	Similarity	Base m/z	Name
1	3.017	23252668	11.77	95	79.05	Pyridine
2	3.135	48940685	24.78	97	77.00	Trifluoroacetamide, TMS derivative
3	3.633	122513518	40.03	92	131.15	1-Butanol, TMS derivative
4	4.893	103773	0.05	86	221.10	Trisiloxane, octamethyl-
5	4.950	1225065	0.62	95	57.05	Isobutyl ether
6	5.747	198133	0.10	93	71.05	4-Heptanone, 3-methyl-
7	6.165	52376	0.03	90	43.05	Propanoic acid, 2-methyl-, butyl ester
8	6.203	22023	0.01	88	71.05	Butanoic acid, 2-methylpropyl ester
9	6.260	605518	0.31	83	87.10	2-Decanone o-methyloxime
10	6.754	23895	8.01	68	149.10	Hexadecanoic acid,methyl ester
11	6.914	255873	12.13	95	71.05	9-Octadecenoic acid,methyl ester
12	7.751	162123	2.08	93	57.05	Tridecane, 6-methyl-
13	25.617	147465	0.07	78	73.05	Thymol-.beta.-d-glucopyranoside, tetrakis(O-trimethylsilyl)-
		197503115	100.00			

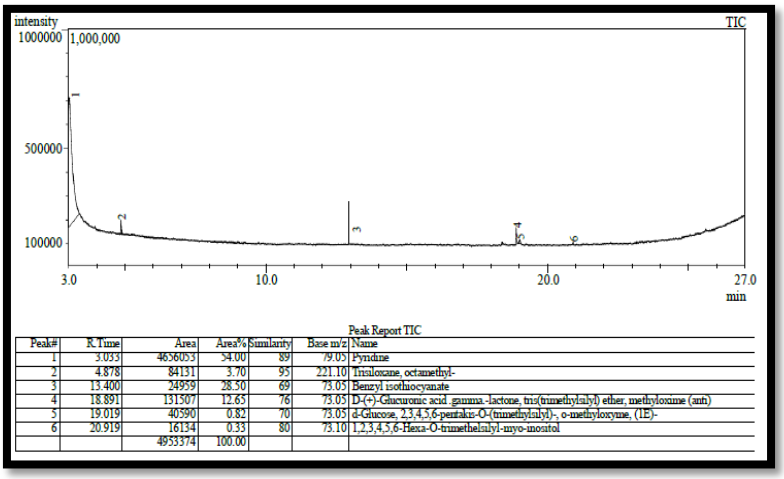
Fig 7(B): Gc-Ms Analysis of Butanolic Extract of Neem



Peak Report TIC

Peak#	R.Time	Area	Area%	Similarity	Base m/z	Name
1	3.022	51657428	18.60	95	77.00	Trifluoroacetamide, TMS derivative
2	4.877	141382	1.23	93	221.10	Trisiloxane, octamethyl-
3	11.346	93290	1.15	92	147.10	Glycerol, 3TMS derivative
4	16.872	44397	0.07	87	73.05	Levoglucosan, 3TMS derivative
5	17.562	18309	0.03	70	217.05	Arabinofuranose, 1,2,3,5-tetrakis-O-(trimethylsilyl)-
6	17.712	80706	0.13	80	217.10	D-(-)-Tagatofuranose, pentakis(trimethylsilyl) ether (isomer 2)
7	18.238	92208	10.15	94	204.10	Ethyl .alpha.-D-glucopyranoside, 4TMS derivative
8	18.769	1329290	12.13	92	73.05	D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-, O-methyloxime
9	18.872	1028782	11.64	91	217.10	Tridecane, 6-methyl
10	18.981	25559	0.04	71	73.05	D-Allose, oxime (isomer 1), 6TMS derivative
11	19.039	1158425	10.85	91	73.05	D-(+)-Talose, pentakis(trimethylsilyl) ether, methyloxime (syn)
12	19.229	214004	0.34	91	73.05	D-Allose, pentakis(trimethylsilyl) ether, methyloxime (syn)
13	19.382	176941	0.28	95	204.10	Ethyl .alpha.-D-glucopyranoside, 4TMS derivative
14	19.541	44065	0.07	85	204.10	Glucose, 5TMS derivative
15	20.913	141787	10.23	94	305.15	1,2,3,4,5,6-Hexa-O-trimethelsilyl-myo-inositol
16	21.395	60772	8.10	86	143.10	Phytol, TMS derivative
17	21.702	104273	0.17	74	73.05	Methyl .alpha.-D-glucofuranoside, 4TMS derivative
18	21.860	83534	0.13	88	204.10	D-(+)-Cellobiose, (isomer 1), 8TMS derivative
19	22.905	34379	0.05	85	204.10	Glyceryl-glycoside 1MS ether
20	24.452	46570	0.07	76	204.10	D-(+)-Cellobiose, (isomer 1), 8TMS derivative
21	24.761	32319	0.05	71	73.05	.alpha.-L-Galactofuranose, 6-deoxy-1,2,3,5-tetrakis-O-(trimethylsilyl)-
22	25.613	5897657	15.43	90	361.15	Sucrose, 8TMS derivative
23	26.301	33912	0.05	70	204.10	D-(+)-Cellobiose, octakis(trimethylsilyl) ether, methyloxime (isomer 1)
		62539969	100.00			

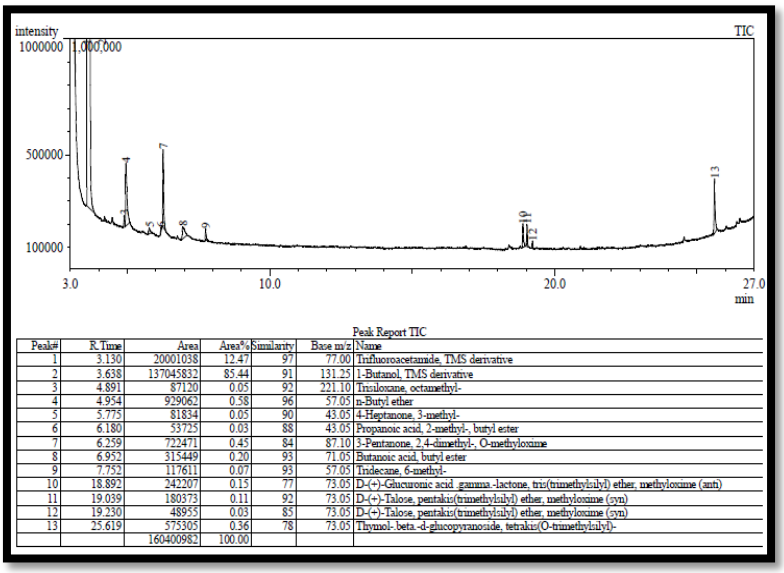
Fig 7(C): Gc-Ms Analysis of Ethyl Acetate Extract of Neem



Peak Report TIC

Peak#	R.Time	Area	Area%	Similarity	Base m/z	Name
1	3.033	4656053	54.00	89	79.05	Pyridine
2	4.878	84131	3.70	95	221.10	Trisiloxane, octamethyl-
3	13.400	24959	28.50	69	73.05	Benzyl isothiocyanate
4	18.891	131507	12.65	76	73.05	D-(+)-Glucuronic acid .gamma.-lactone, tris(trimethylsilyl) ether, methyloxime (anti)
5	19.019	40590	0.82	70	73.05	d-Glucose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, o-methyloxyme, (1E)-
6	20.919	16134	0.33	80	73.10	1,2,3,4,5,6-Hexa-O-trimethelsilyl-myo-inositol
		4953374	100.00			

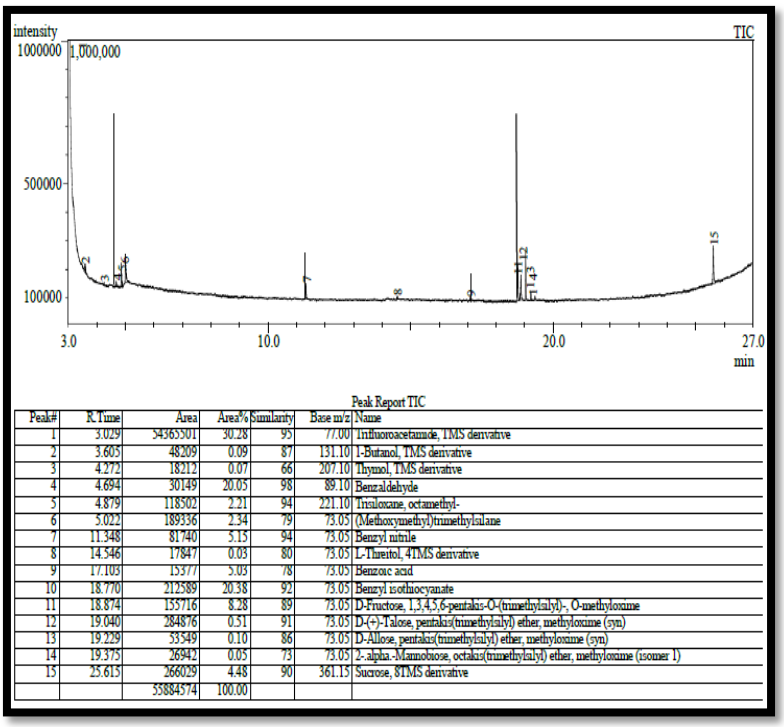
Fig 8(A): Gc-Ms Analysis of Ethanolic Extract of Miswak



Peak Report TIC

Peak#	R.Time	Area	Area%	Similarity	Base m/z	Name
1	3.130	20001038	12.47	97	77.00	Trifluoroacetamide, TMS derivative
2	3.638	137045832	85.44	91	131.25	1-Butanol, TMS derivative
3	4.891	87120	0.05	92	221.10	Trisiloxane, octamethyl-
4	4.954	929062	0.58	96	57.05	n-Butyl ether
5	5.775	81834	0.05	90	43.05	4-Heptanone, 3-methyl-
6	6.180	55725	0.03	88	43.05	Propanoic acid, 2-methyl-, butyl ester
7	6.259	722471	0.45	84	87.10	3-Pentanone, 2,4-dimethyl-, O-methyloxime
8	6.952	315449	0.20	93	71.05	Butanoic acid, butyl ester
9	7.752	117611	0.07	93	57.05	Tridecane, 6-methyl-
10	18.892	242207	0.15	77	73.05	D-(+)-Glucuronic acid .gamma.-lactone, tris(trimethylsilyl) ether, methyloxime (anti)
11	19.039	180373	0.11	92	73.05	D-(+)-Talose, pentakis(trimethylsilyl) ether, methyloxime (syn)
12	19.230	48955	0.03	85	73.05	D-(+)-Talose, pentakis(trimethylsilyl) ether, methyloxime (syn)
13	25.619	575305	0.36	78	73.05	Thymol- beta.-d-glucopyranoside, tetrakis(O-trimethylsilyl)-
		160400982	100.00			

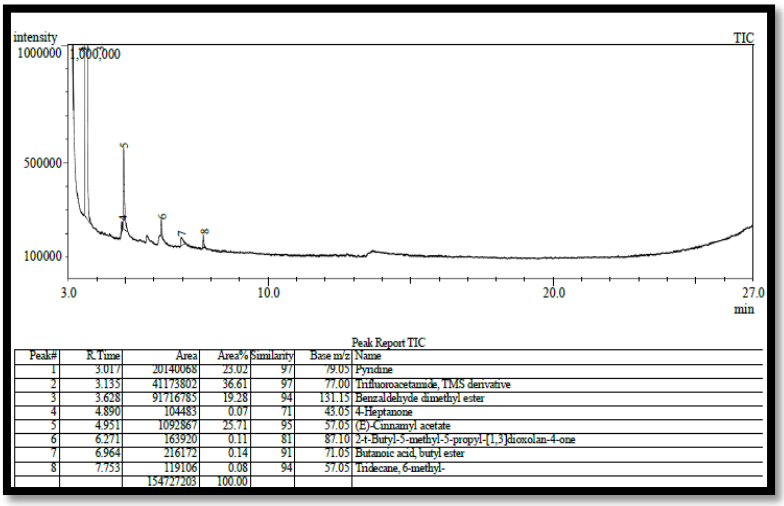
Fig 8(B): Gc-Ms Analysis of Butanolic Extract of Miswak



Peak Report TIC

Peak#	R.Time	Area	Area%	Similarity	Base m/z	Name
1	3.029	54365501	30.28	95	77.00	Trifluoroacetamide, TMS derivative
2	3.605	48209	0.09	87	131.10	1-Butanol, TMS derivative
3	4.272	18212	0.07	66	207.10	Thymol, TMS derivative
4	4.694	30149	20.05	98	89.10	Benzaldehyde
5	4.879	118502	2.21	94	221.10	Trisiloxane, octamethyl-
6	5.022	189336	2.34	79	73.05	(Methoxymethyl)trimethylsilane
7	11.348	81740	5.15	94	73.05	Benzyl nitrile
8	14.546	17847	0.03	80	73.05	L-Threitol, 4TMS derivative
9	17.103	15377	5.03	78	73.05	Benzoic acid
10	18.770	212589	20.38	92	73.05	Benzyl isothiocyanate
11	18.874	155716	8.28	89	73.05	D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-, O-methyloxime
12	19.040	284876	0.51	91	73.05	D-(+)-Talose, pentakis(trimethylsilyl) ether, methyloxime (syn)
13	19.229	53549	0.10	86	73.05	D-Allose, pentakis(trimethylsilyl) ether, methyloxime (syn)
14	19.375	26942	0.05	73	73.05	2-.alpha.-Mannobiose, octakis(trimethylsilyl) ether, methyloxime (isomer 1)
15	25.615	266029	4.48	90	361.15	Sucrose, 8TMS derivative
		55884574	100.00			

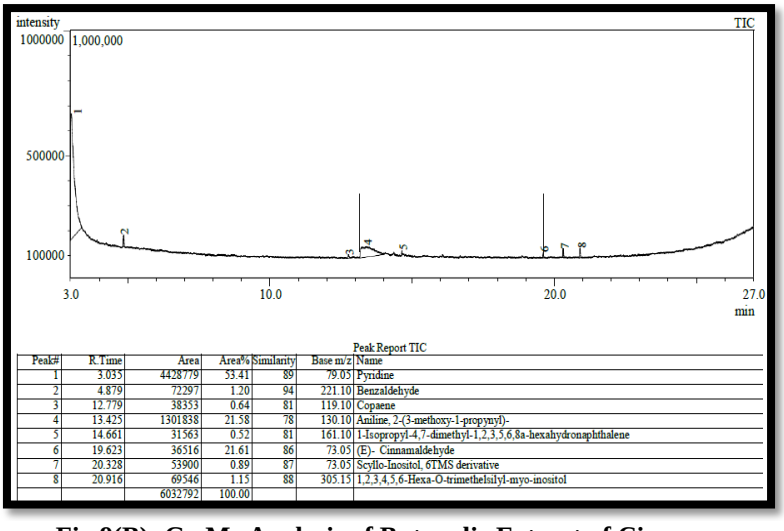
Fig 8(C): Gc-Ms Analysis of Ethyl Acetate Extract of Miswak



Peak Report TIC

Peak#	R.Time	Area	Area%	Similarity	Base m/z	Name
1	3.017	20140068	23.02	97	79.05	Pyridine
2	3.135	41175802	36.61	97	77.00	Trifluoroacetamide, TMS derivative
3	3.628	91716785	19.28	94	131.15	Benzaldehyde dimethyl ester
4	4.890	104483	0.07	71	43.05	4-Heptanone
5	4.951	1092867	25.71	95	57.05	(E)-Cinnamyl acetate
6	6.271	163920	0.11	81	87.10	2-t-Butyl-5-methyl-5-propyl-[1,3]dioxolan-4-one
7	6.964	216172	0.14	91	71.05	Butanoic acid, butyl ester
8	7.753	119106	0.08	94	57.05	Tridecane, 6-methyl-
		154727203	100.00			

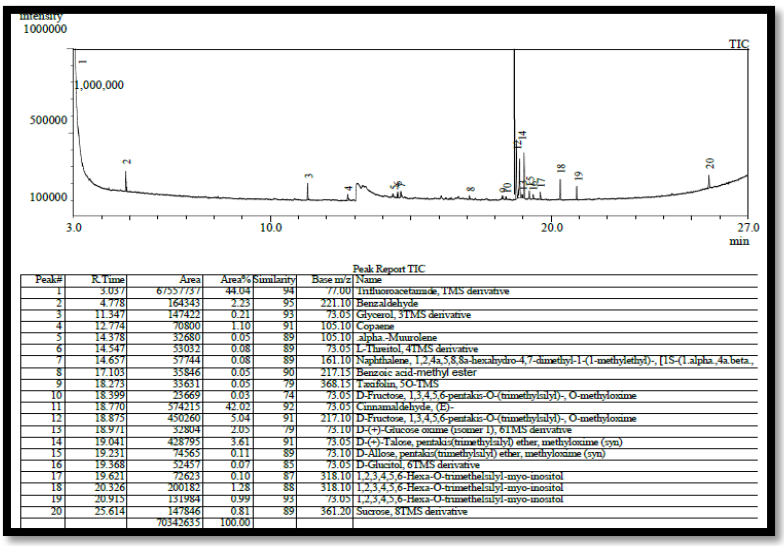
Fig 9(A): Gc-Ms Analysis of Ethanolic Extract of Cinnamon



Peak Report TIC

Peak#	R.Time	Area	Area%	Similarity	Base m/z	Name
1	3.035	4428779	53.41	89	79.05	Pyridine
2	4.879	72297	1.20	94	221.10	Benzaldehyde
3	12.779	38353	0.64	81	119.10	Copaene
4	13.425	1301838	21.58	78	130.10	Aniline, 2-(3-methoxy-1-propynyl)-
5	14.661	31563	0.52	81	161.10	1-Isopropyl-4,7-dimethyl-1,2,3,5,6,8a-hexahydronaphthalene
6	19.623	36516	21.61	86	73.05	(E)- Cinnamaldehyde
7	20.328	53900	0.89	87	73.05	Scyllo-Inositol, 6TMS derivative
8	20.916	69546	1.15	88	305.15	1,2,3,4,5,6-Hexa-O-trimethelsilyl-myo-inositol
		6032792	100.00			

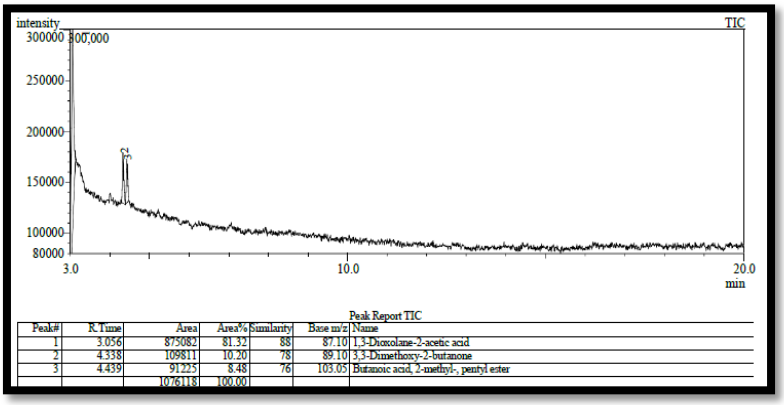
Fig 9(B): Gc-Ms Analysis of Butanolic Extract of Cinnamon



Peak Report TIC

Peak#	R.Time	Area	Area%	Similarity	Base m/z	Name
1	3.037	6755777377	44.04	94	77.00	Trifluoroacetimide, TMS derivative
2	4.778	164343	2.23	95	221.10	Benzaldehyde
3	11.347	147422	0.21	93	73.05	Glycerol, 3TMS derivative
4	12.774	70800	1.10	91	105.10	Copaene
5	14.378	32680	0.05	89	105.10	alpha.-Muurolene
6	14.547	53032	0.08	89	73.05	L-Threitol, 4TMS derivative
7	14.657	57744	0.08	89	161.10	Naphthalene, 1,2,4a,5,8,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, [1S-(1.alpha.,4a.beta.,
8	17.103	35846	0.05	90	217.15	Benzoic acid-methyl ester
9	18.273	33631	0.05	79	368.15	Taxifolin, 5O-TMS
10	18.399	23669	0.03	74	73.05	D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-, O-methyloxime
11	18.770	574215	42.02	92	73.05	Cinnamaldehyde, (E)-
12	18.875	450260	5.04	91	217.10	D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-, O-methyloxime
13	18.971	22804	2.05	79	73.10	D-(+)-Lactose oxime (isomer 1), 6TMS derivative
14	19.041	428795	3.61	91	73.05	D-(+)-Talose, pentakis(trimethylsilyl) ether, methyloxime (syn)
15	19.231	74565	0.11	89	73.10	D-Allose, pentakis(trimethylsilyl) ether, methyloxime (syn)
16	19.368	52457	0.07	85	73.05	D-Glucitol, 6TMS derivative
17	19.621	72623	0.10	87	318.10	1,2,3,4,5,6-Hexa-O-trimethelsilyl-myo-inositol
18	20.326	200182	1.28	88	318.10	1,2,3,4,5,6-Hexa-O-trimethelsilyl-myo-inositol
19	20.915	131984	0.99	93	73.05	1,2,3,4,5,6-Hexa-O-trimethelsilyl-myo-inositol
20	25.614	147846	0.81	89	361.20	Sucrose, 8TMS derivative
		70342635	100.00			

Fig 9(C): Gc-Ms Analysis of Ethyl Acetate Extract of Cinnamon



Peak Report TIC

Peak#	R.Time	Area	Area%	Similarity	Base m/z	Name
1	3.056	875082	81.32	88	87.10	1,3-Dioxolane-2-acetic acid
2	4.338	109811	10.20	78	89.10	3,3-Dimethoxy-2-butanone
3	4.439	91225	8.48	76	103.05	Butanoic acid, 2-methyl-, pentyl ester
		1076118	100.00			

Fig 10: Gc-Ms Analysis of Apple Cider Vinegar

B. ANTIMICROBIAL SUSCEPTIBILITY TEST- AGAR WELL DIFFUSION ASSAY

a) Neem Extracts

Various concentration of 200 mg/mL, 100 mg/mL and 50 mg/mL of ethanolic, butanolic and ethyl acetate extracts of neem were subjected to agar well diffusion test in which only butanolic extract of neem showed significant antimicrobial activity whereas the ethanolic and ethyl acetate extracts of neem did not show any activity. Thus, the antimicrobial activity was performed by raising the concentration to 500 mg/mL at which ethanolic and ethyl acetate extracts of neem also showed significant antimicrobial activity.

A significant difference among the zones of inhibition of ethanolic, butanolic and ethyl acetate extracts of neem was seen out of which butanolic extract of neem showed significantly larger mean zone of inhibition against *E. faecalis*. ($p < 0.001$) [Fig.11] [Table 1] [Graph 1]

b) Miswak Extracts

Various concentration of 200 mg/mL, 100 mg/mL and 50 mg/mL of ethanolic, butanolic and ethyl acetate extracts of miswak, were subjected to agar well diffusion test in which only ethyl acetate extracts of miswak showed significant antimicrobial activity whereas the ethanolic and butanolic extracts of miswak did not show any activity. Thus, the antimicrobial activity was performed by raising the concentration to 500 mg/mL at which ethanolic and butanolic extracts of miswak also showed significant antimicrobial activity.

A significant difference among the zones of inhibition of ethanolic, butanolic and ethyl acetate extracts of miswak was seen out of which ethyl acetate extract of miswak showed maximum zone of inhibition against *E. faecalis*. ($p < 0.001$) [Fig. 12] [Table 2] [Graph 2]

c) Cinnamon Extracts

Various concentration of 200 mg/mL, 100 mg/mL and 50 mg/mL of ethanolic, butanolic and ethyl acetate extracts of cinnamon, were subjected to agar well diffusion test. A significant difference among the zones of inhibition of ethanolic,

butanolic and ethyl acetate extracts of cinnamon was seen out of which ethyl acetate extract of cinnamon showed maximum zone of inhibition against *E. faecalis*. ($p < 0.001$) [Fig. 13] [Table 3] [Graph 3]

d)Phytomedicinal Extracts

On comparing most potent extracts of neem, miswak and cinnamon with apple cider vinegar, a significant difference among tested extracts was found such that, ethyl acetate extract of cinnamon (50 mg/mL) showed highest mean zone of inhibition against *E.faecalis* followed by ethyl acetate extract of miswak (50 mg/mL), apple cider vinegar and butanolic extract of neem (50 mg/mL).[Fig. 14] [Table 4]

e) Chemical Root Canal Irrigants

A significant difference was found among chemical root canal irrigants, where highest mean zone of inhibition against *E.faecalis* was seen by QMix followed by 3% sodium hypochlorite. Negative controls i.e. saline and DMSO did not show antimicrobial activity against *E.faecalis*. [Fig. 15]

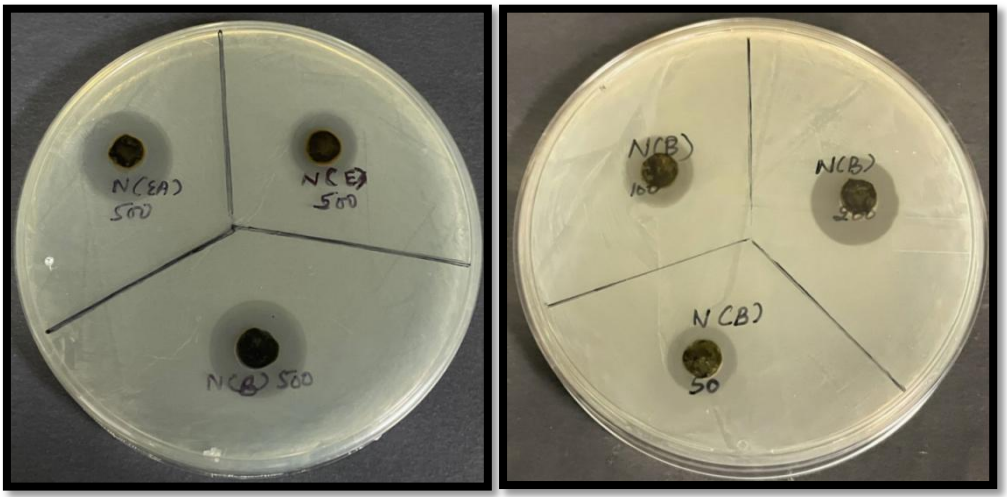
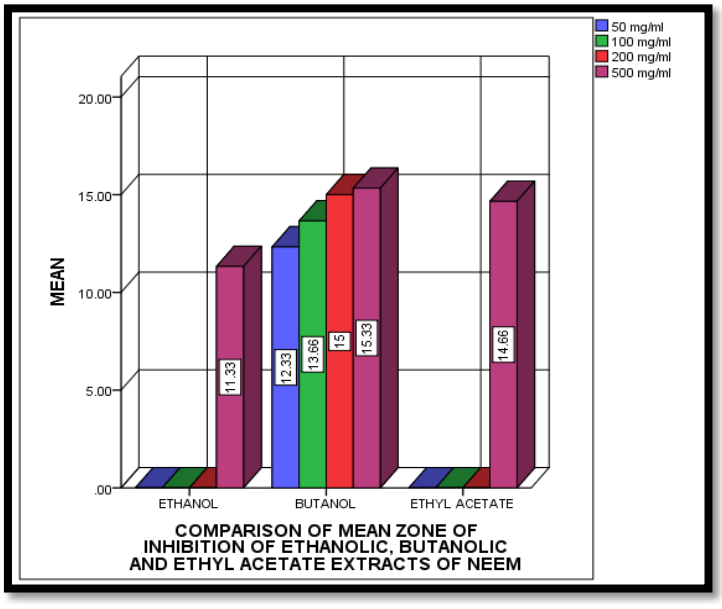


Fig 11:Zone of Inhibition of Ethanolic (E), Butanolic (B) and Ethyl Acetate (Ea) Extracts of Neem

Table 1: Comparison of Mean Zone of Inhibition of Ethanolic, Butanolic and Ethyl Acetate Extracts of Neem

GROUPS (NEEM)	CONCENTRATION (MEAN ±SD)								N
	50 mg/mL		100 mg/mL		200 mg/mL		500 mg/Ml		
	M	SD	M	SD	M	SD	M	SD	
ETHANOL	0	0	0	0	0	0	11.33	0.57	3
BUTANOL	12.33	0.57	13.66	0.57	15	0	15.33	1.15	3
ETHYL ACETATE	0	0	0	0	0	0	14.66	0.57	3
P-VALUE	0.001*								



Graph 1: Comparison of Mean Zone of Inhibition of Ethanolic, Butanolic and Ethyl Acetate Extracts of Neem

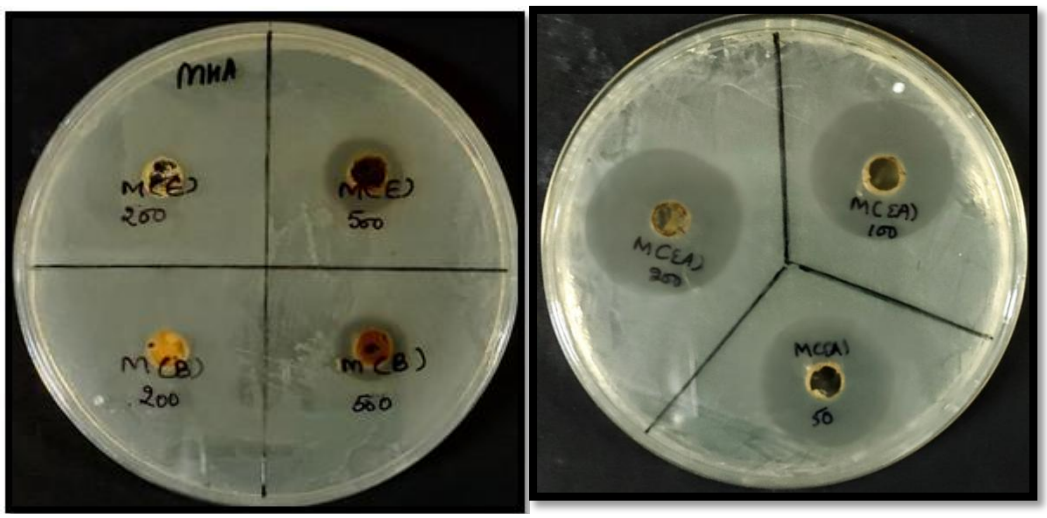
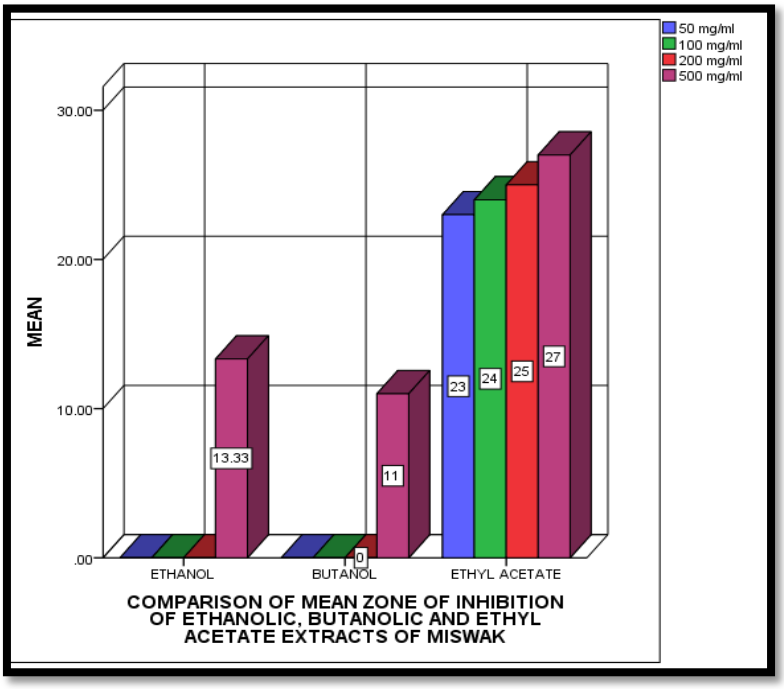


Fig 12: Zone of Inhibition of Ethanolic (E), Butanolic (B) and Ethyl Acetate (Ea) Extracts of Miswak

Table 2: Comparison of Mean Zone of Inhibition of Ethanolic, Butanolic and Ethyl Acetate Extracts of Miswak

GROUPS (MISWAK)	CONCENTRATION (MEAN ±SD)								N
	50 mg/mL		100 mg/mL		200 mg/mL		500 mg/mL		
	M	SD	M	SD	M	SD	M	SD	
ETHANOL	0	0	0	0	0	0	13.33	0.57	3
BUTANOL	0	0	0	0	0	0	11	0	3
ETHYL ACETATE	23	0	24	0	25	0	27	1	3
P-VALUE	0.001*								



Graph 2: Comparison of Mean Zone of Inhibition of Ethanolic, Butanolic and Ethyl Acetate Extracts of Miswak

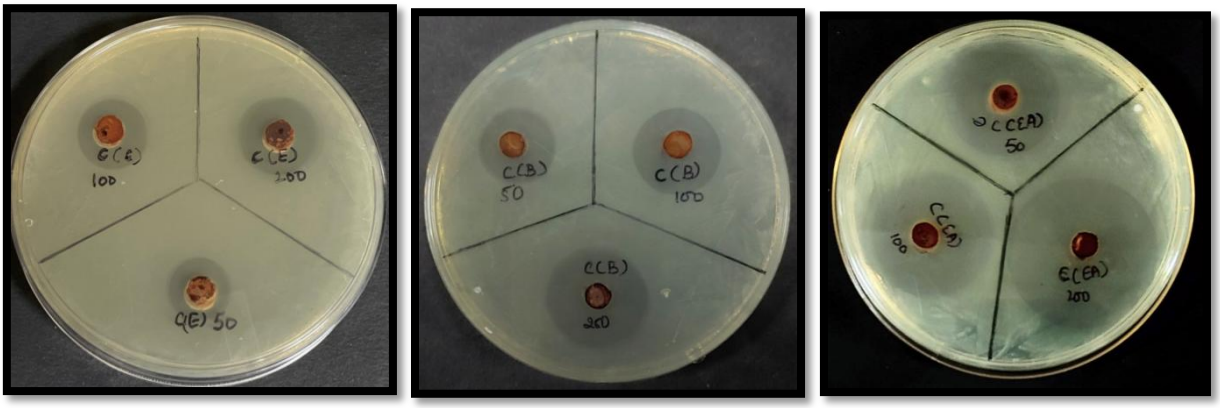
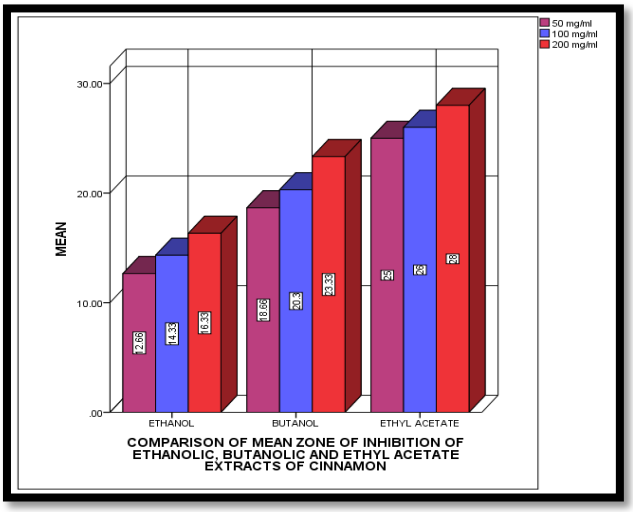


Fig 13: Zone of Inhibition of Ethanolic (E), Butanolic (B) and Ethyl Acetate (Ea) Extracts of Cinnamon

Table 3: Comparison of Mean Zone of Inhibition of Ethanolic, Butanolic and Ethyl Acetate Extracts of Cinnamon

GROUPS (CINNAMON)	CONCENTRATION (MEAN ±SD)						N
	50 mg/mL		100 mg/mL		200 mg/Ml		
	M	SD	M	SD	M	SD	
ETHANOL	12.66	1.15	14.33	0.57	16.33	0.57	3
BUTANOL	18.66	0.57	20.3	0.57	23.33	0.57	3
ETHYL ACETATE	25	0	26	0	28	0	3
P-VALUE	0.001*						



Graph 3: Comparison of Mean Zone of Inhibition of Ethanolic, Butanolic and Ethyl Acetate Extracts of Cinnamon

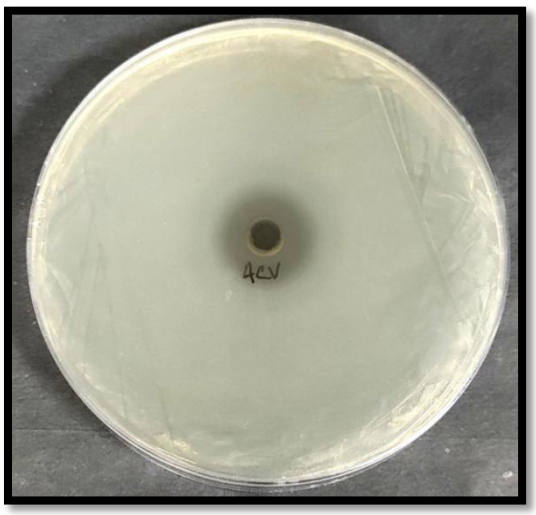


Fig 14: Zone of Inhibition of Apple Cider Vinegar

Table 4: Comparison of Mean Zone of Inhibition of Phytomedicinal Extracts

GROUPS	CONCENTRATION	MEAN (mm)	SD
NEEM (B)	50 mg/mL	12.33	0.57
MISWAK (EA)	50 mg/mL	23	0
CINNAMON (EA)	50 mg/mL	25	0
APPLE CIDER VINEGAR	100%	16	1
P-VALUE	0.001*		

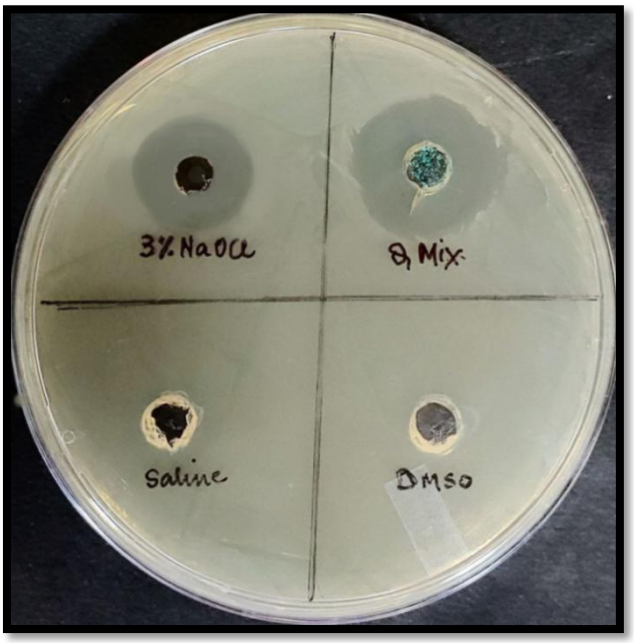


Fig 15:Zone of Inhibition of Chemical Irrigants

F. INTER GROUP COMPARISON OF MEAN ZONE OF INHIBITION OF ALL TEST IRRIGANTS

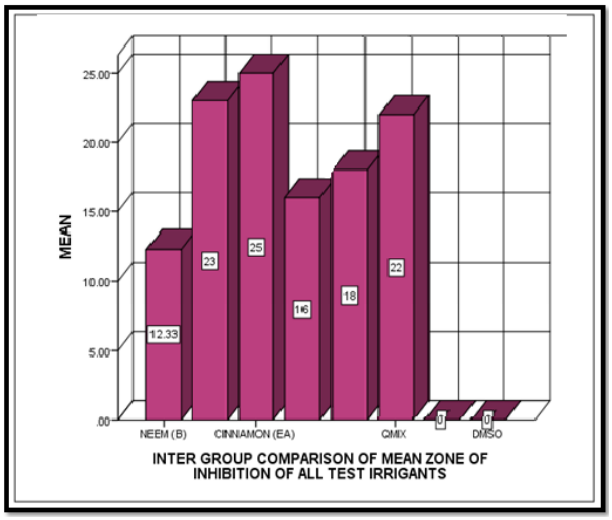
On comparing all the groups, significant difference was found among all groups. It was found that ethyl acetate extract of cinnamon showed highest mean zone of inhibition against *E.faecalis*followed by ethyl acetate extract of miswak, QMix,3% sodium hypochlorite, apple cider vinegar and butanolic extract of neem. [Table 5] [Graph 4]

G. INTRA GROUP COMPARISON OF MEAN ZONE OF INHIBITION OF ALL TEST IRRIGANTS

Intra group comparison of mean zone of inhibition of all test irrigants shows significant difference between all groups. However, no significant difference was seen between normal saline and DMSO. [Table 6]

Table 5: Inter Group Comparison of Mean Zone of Inhibition of All Test Irrigants

GROUPS	MEAN (mm)	SD	MIN	MAX
NEEM (B)50 mg/Ml	12.33	0.57	12	13
MISWAK (EA)50 mg/mL	23	0	23	23
CINNAMON (EA)50 mg/mL	25	0	25	25
APPLE CIDER VINEGAR100%	16	1	15	17
3% SODIUM HYPOCHLORITE	18	0	18	18
QMIX	22	0	22	23
SALINE	0	0	0	0
DMSO	0	0	0	0
P-VALUE	0.001*			



Graph 4: Inter Group Comparison of Mean Zone of Inhibition of All Test Irrigants

Table 6: Intra Group Comparison of Mean Zone of Inhibition of All Test Irrigants

COMPARISON	GROUPS	P- VALUE
NEEM (B)	MISWAK (EA)	0.001*
	CINNAMON (EA)	0.001*
	APPLE CIDER VINEGAR	0.001*
	3 % SODIUM HYPOCHLORITE	0.001*
	QMIX	0.001*
	SALINE	0.001*
	DMSO	0.001*
MISWAK (EA)	CINNAMON (EA)	0.001*
	APPLE CIDER VINEGAR	0.001*
	3 % SODIUM HYPOCHLORITE	0.001*
	QMIX	0.001*
	SALINE	0.001*
	DMSO	0.001*
CINNAMON (EA)	APPLE CIDER VINEGAR	0.001*
	3 % SODIUM HYPOCHLORITE	0.001*
	QMIX	0.001*
	SALINE	0.001*
	DMSO	0.001*
APPLE CIDER VINEGAR	3 % SODIUM HYPOCHLORITE	0.001*
	QMIX	0.001*
	SALINE	0.001*
	DMSO	0.001*
3 % SODIUM HYPOCHLORITE	QMIX	0.001*
	SALINE	0.001*
	DMSO	0.001*
QMIX	SALINE	0.001*
	DMSO	0.001*
SALINE	DMSO	0.001*

DISCUSSION

The plant products used in the study are neem, miswak, cinnamon, apple cider vinegar. The selection of the tested plants was based on their antimicrobial efficacy reported in literature [11,12].

Preparation of medicinal plants for experimental purposes is an initial step and key in achieving quality research outcome. Extraction, is separating the medicinally active constituents contained inside plant materials using selective solvents through a standard procedure. The plant material were dissolved in three different solvents i.e. ethanol, butanol and ethyl acetate. This was done due to difference in solubility of the active components in different organic solvents. The plant extracts were concentrated to dryness under reduced pressure using rota evaporator to give the dry fractions of each solvent [13,14].

Gas chromatography-mass spectroscopy (GC-MS) is a combined analytical technique used to determine and identify compounds present in a plant sample. GC-MS plays an essential role in the phytochemical analysis and chemotaxonomic studies of medicinal plants containing biologically active components. GC-MS analysis was performed to evaluate the possible biologically active phytochemical constituents in the plant extracts [15,16].

Various concentrations ranging from 50 to 200 mg/mL for plant extracts, commercially available 100% apple cider vinegar were tested based on previous studies [17,18].

For preparation of test solutions, crude plant extracts were dissolved in DMSO for antimicrobial evaluation and endodontic irrigation. DMSO was used as a solvent, as it does not have any antibacterial action of its own, which had been proved in previous studies and in this study by using it as a control. It is a highly polar organic reagent that has exceptional solvent properties for organic and inorganic chemicals. Thus, it helps in complete dissolution of extracts and brings out the properties of all the components of herb being dissolved [19].

Agar well diffusion method is widely used to evaluate the antimicrobial activity of plants. So, this was used to compare the zone of inhibition produced by the antimicrobial agents used in this study. The advantage of this method is that, it allows direct comparison of the materials against the organisms, indicating which material has the potential to eliminate bacteria. However, the limitation of this method is that the result not only depend on the toxicity of the material for the particular organism, but is also influenced by the ability of the material to diffuse across the medium [9].

Mueller-Hinton agar culture medium was used in this study, since it is widely used in sensitivity tests and commonly used to culture *E. faecalis* [10]. The most potent extract among ethanolic, butanolic and ethyl acetate extracts of neem, miswak and cinnamon were identified and used further in the study for comparison with chemical root canal irrigants. The most potent extracts were butanolic extract of neem, ethyl acetate extract of miswak and ethyl acetate extract of cinnamon.

The ethyl acetate extract of cinnamon showed highestantibacterial effectiveness i.e. highest mean zone of inhibition against *E.faecalis* followed by ethyl acetate extract of miswak, QMix, 3% sodium hypochlorite, apple cider vinegar and butanolic extract of neem.

Neem extract has shown a significant antibacterial action against *E. faecalis*. Neem extract is rich in antimicrobial phytoconstituents such as alkaloids, glycosides flavonoids, phenolic compounds, steroids, triterpenoids, carotenoids, and tetra-triterpenoids. In this study, the GC–MS analysis of neem revealed the presence of 9-octadecenoic acid, hexadecanoic acid, tridecane, 6-methyl-ester, phytol, thymol, etc which have also beenreported previously to possess antibacterial activity [20,21]. Butanolic extract of neem showed greater zone of inhiition than ethanolic and ethyl acetate extract of neem. Similar results were found by Hossain et al who evaluated antioxidant capacity of different crude extracts of neem and showed butanolic extract to be more efficacious than ethyl acetate extract of neem [22].Sundaram et al, Ambareen et al in their study showed significant antimicrobial activity of neem against *E. faecalis* but less than that of sodium hypochlorite irrigant [23,24]. Also, Shetty et al demonstrated QMix to be a more effective root canal irrigant when compared to neem [25]. These results are in accordance to the findings of present study. While Kini et al and Sinha et al in their study showed similar antibacterial effectiveness of neem when compared to sodium hypochlorite [26,27].This may be attributed to difference in method of preparation of extract.

Miswak sticks have been widely used in past as an oral hygiene aid. Results of gas chromatography- mass spectrometry analysis of miswak extracts in this study shows the presence of various phytomedicinal components benzyl isothiocyanate, *n*-benzyloctadecenamide , n-hexadecanoic acid, benzoic acid, benzyl isocyanate, benzyl nitrile, thymol ,etc. Sofrata et al suggests presence of benzyl isothiocyanate in miswak to be associated with its high antimicrobial efficacy [28]. The results of present study were in accordance to the study by Sabawi et al who demonstrated that miswak showed high antimicrobial activity against *E. faecalis* compared to NaOCI and CHX [29]. Also, Ethyl acetate fraction of miswak showed the significantly higher antimicrobial activity when compared to other fractions of plant extract in a study by Radwan et al. which is in accordance to our study [30].

Cinnamon extract has recently gained a limelight as an irrigant in endodontics due its antibacterial properties. Gas chromatography- mass spectrometry analysis of cinnamon in this study identified various phytomedicinal compounds from cinnamon such as (E)-cinnamaldehyde, benzaldehyde, copaene, naphthalene, benzoic acid-methyl ester, etc. Hameed et al suggests that *Cinnamomum zeylanicum* possesses remarkable antimicrobial activity, which is mainly due to (E)-cinnamaldehyde [31].The present study shows cinnamon extract irrigant to have highest antibacterial effectiveness among herbal and chemical irrigants used in this study. Panchal V. et al in her study also concluded that cinnamon extract irrigant showed better reduction in *E. faecalis* as compared to 3% sodium hypochlorite and neem extract irrigant [18]. Mahdi et al also showed higher efficacy of cinnamon as compared to sodium hypochlorite against *E. faecalis* [32].

Very few studies have been carried out using apple cider vinegar as a potential root canal irrigant. Gas chromatography- mass spectrometry analysis of Apple cider vinegar in this study shows the presence of various

phytoactive components like 1,3-dioxolane-2-acetic acid, 3,3-dimethoxy-2-butanone , butanoic acid, 2-methyl-pentyl ester etc. Our results are in accordance to Saquib A et al who suggests that antimicrobial activity of apple cider vinegar is mainly due to presence of various organic acids especially acetic acid, making it an effective bactericide and fungicide [33].Truta et al also identified similar phytochemicals in apple cider vinegar as found in this study [34]. Mohanty S et al has also shown significant antimicrobial activity of Apple cider vinegar against *E. faecalis* as shown in our study [35].

QMix root canal irrigant has shown significant antimicrobial activity against *E. faecalis*. Our study is in accordance to findings of study done by Lim et al in 2020 that showed QMix has higher antibacterial activity than NaOCl with lower concentration [36]. The results of this study differed from Ye WH et al, where sodium hypochlorite outperformed QMix in its antibacterial activity because the concentration used was much higher i.e. 6% [37].

A significant difference in antimicrobial efficacy was seen in all the groups when compared to saline and DMSO. Saline and DMSO did not show any antimicrobial activity against *E. faecalis.*Similar results were seen in a study by Shingare et al [38].

The present study is thus a preliminary study to assess and compare the antibacterial effectiveness of phytomedicinal irrigants with chemical irrigants. There are several limitations of the present study such as, it is an in vitro study in which the exact oral conditions could not be simulated. Also, the present values were measured under ideal laboratory conditions which may not necessarily be expected in the clinical setup.

CONCLUSION

Within the limitations of the present in vitro study, based on the employed methodology and according to the results obtained, it was concluded that

1) All phytomedicinal extracts showed significant antimicrobial activity against *E. faecalis.*
2) Cinnamon showed highest antimicrobial activity i.e. highest mean zone of inhibition against *E. faecalis* among all the tested irrigants.
3) The order of highest to lowest antibacterial effectiveness against *E. faecalis* was cinnamon followed by miswak, QMix, 3% sodium hypochlorite, apple cider vinegar, neem and saline.

Hence, phytomedicinal irrigants have huge potential to be used as root canal irrigant, an alternative to chemical root canal irrigants.

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