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ANTIBACTERIAL ACTIVITY OF *EUCALYPTUS GLOBULUS* ESSENTIAL OIL FRACTIONS AGAINST ANTIBIOTIC RESISTANT *ENTEROCOCCUS FAECIUM* ISOLATED FROM DIARRHEIC SHEEP

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ABSTRACT

Antibiotic resistant *Enterococcus faecium* isolated from the diarrheic sheep were subjected to antibacterial activity of plant essential oils and their fractions. The fraction with least minimum inhibitory concentration (MIC) was subjected to cytotoxicity and gas chromatography-mass spectrometry (GCMS) analysis. Primarily, biochemically characterized isolates were confirmed by PCR amplification of 16S rRNA gene followed by nucleotide sequencing and accession numbers received were MW332526.1, MW332527.1, MW332528.1 and MW332529.1. These isolates were screened for antibiotic resistance to a variety of antibiotic classes and recorded as resistant to all the tested antibiotics. Antibacterial activity of plant essential oils (n=05) was checked against three selected antibiotic resistant *E. faecium* isolates. Activity of *Eucalyptus globulus* was highest (13.00±1.3 mm) and among the fractions of *E. globulus,* n-hexane plus chloroform depicted a higher mean zone of inhibition (13.45±1.11mm) with least MIC (25.28±7.41 mg/mL) which differed significantly (P<0.05) with other fractions tested. Cell survival percentage was 58.60 at 112.10 mg/mL concentration. GCMS analysis revealed that the highest percentage (14.4%) was of Bicyclo[2.2.1]heptan-2-ol,1,7,7-trimethyl. It was concluded that *E. globulus* fraction n-hexane plus chloroform showed significantly (P<0.05) better results as compared to other (n=08) *E. globulus* solvent fractions for treatment of antibiotic resistant cases of diarrhea in sheep caused by *E. faecium*.

Keywords: Antibiotic resistance, *Enterococcus faecium*, *Eucalyptus globulus* fractions, Cell survival percentage, Gas chromatography-mass spectrometry.

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INTRODUCTION

Invention of antimicrobials provoked the thought to control pathogens since early 1900 but due to misuse of antibiotics and adaptation of antimicrobial resistance lead to universal dilemma to control microbial infections (Ambrosio *et al*., 2017). *E. faecium* is primarily important emerging pathogen causing nosocomial infection. *E. faecium* and *E. faecalis* are often side by side in nosocomial infections but *E. faecium* have more pathogenicity, prevalence and mortality rate than *E. faecalis*. *E. faecium* is found more in blood cultures than urine, skin and other genital specimens. *E. faecium* has acquired antibiotic resistance against number of antibiotics specifically vancomycin giving rise to Van A type ,Van B type and Van D type (Klare *et al*., 2003). Moreover, *E. faecium* is emerging as nosocomial and opportunistic pathogen so it is required to find alternatives for the control of such infections (Darroui-Mokaddem *et al*., 2010).

To fill this gap, various medicinal herbal extracts are used to check their effectiveness against antibiotic resistant bacterial strains (Damjanović-Vratnica *et al*., 2011). Essential oils are preferable over synthetic drugs due to presence of least resistance against them. Essential oils of *E. globulus* among different medicinal plants work effectively against antibiotic resistant *E. faecium*. Moreover, they are safe to be used *in-vivo* as compared to synthetic drugs. Essential oil of *E. globulus* provides not only antibacterial but also antifungal, analgesic, antiseptic, and anti-inflammatory effect (Mulyaningsih *et al*., 2010).

Among all active agents of essential oil of *E. globulus* analyzed by GC-MS; 1, 8-cineole and

aromadendrene comprises high concentration (Mittal, *et al*., 2019). Antimicrobial drugs or herbal medicines are considered effective when they work best on principle of selective toxicity (Akolade *et al*., 2012). Selective toxicity comprises two factors; one determines effectiveness of active agents against pathogen and second is its toxicity against host cells. Hence, cytotoxicity must be checked prior to declare essential oil effective against pathogen. *Eucalyptus globulus* essential oil provides minimal cytotoxicity when checked in vitro on Vero/BHK-21 cell line (Khazraei *et al*., 2021). The Present study was designed to characterize the *E. faecium* isolated from the diarrheic sheep at molecular level followed by evaluation of antimicrobial resistance pattern and plant essential oil as drug alternatives.

MATERIALS AND METHODS

Characterization of *E. faecium:* *E. faecium* (n=04) isolates from diarrheic sheep, were procured from the Institute of Microbiology, UVAS, Lahore. These isolates were previously characterized through biochemical profile. These isolates were revived from microbeads stock and cultured on nutrient agar and incubated aerobically at 37°C for 24 hours. Bacterial microscopic appearance and Gram characteristics were confirmed by Gram's staining. Deoxyribose Nucleic Acid (DNA) was extracted by following the manufacturer recommendations of DNA extraction kit (GeneAll® Exgene™). The extracted DNA was visually confirmed by agarose gel electrophoresis using 0.8% agarose gel. Isolates were confirmed by polymerase chain reaction (PCR) using 16S rRNA gene specific primers (forward primer: 5'-AGTTTGATCCTGGCTCAG-3'; reverse primer: 5'-GTGTGTACAAGGCCCGGGAAC-3') having 1500bp band size following the method of (Ali *et al*., 2022). Reaction mixture was prepared for 25 µL of final volume. PCR amplification was carried out at 95°C for 5 min, followed by 30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min and final extension at 72°C for 10 min. Amplified products were confirmed through agarose gel electrophoresis using 1.5% agarose gel. The amplicons were processed for sequencing and FASTA files were retrieved. Phylogenetic analysis was performed using MEGA X software followed by submission of sequences to NCBI GenBank.

Antibiotic Susceptibility Testing: Molecularly confirmed isolates were subjected to antibiotic susceptibility testing by Kirby-Bauer method following the guidelines provided by clinical and laboratory standards institute (CLSI-2020) (Wayne 2020). Commercially available antibiotic disks of four antibiotics classes' viz., penicillin, macrolides, vancomycin and fluoroquinolones were used for testing. Diameter of zone of inhibition was measured in millimeter (mm).

Activity of essential oils against antibiotic resistant *E. faecium:* Isolates of *E. faecium* (n=03) resistant to multiple antibiotics were subjected to antibacterial activity of commercially available plant essential oils (n=05) including *Elettaria cardamomum, Allium sativum, Cuminum cyminum, Ferula assa-foetida* and *E. globulus* by agar well diffusion method. The antibacterial activity was determined by measuring the zone of inhibition in mm around the wells (Adnan 2019).

Activity of *Eucalyptus globulus* essential oil fractions: *E. globulus* oil fractionation was carried out using different solvents (Rana *et al*., 2011). Oil fractions were prepared in n-Hexane, n-Hexane plus chloroform, chloroform, chloroform plus ethyl acetate, ethyl acetate, ethyl acetate plus methanol, methanol, methanol plus acetonitrile and acetonitrile. Antimicrobial activity was checked through agar well diffusion assay and MIC assay was performed to evaluate the inhibition of visible bacterial growth in broth with minimum amount of essential oils fractions by following the guidelines of CLSI (Nissen *et al*., 2010).

Cytotoxicity and GCMS analysis of *E. globulus* essential oil fractions: Cytotoxicity of essential oils fractions was assessed in Baby hamster kidney-21 (BHK-21) cell line grown in Glasgow minimum essential medium (GMEM) supplemented with 8-10% fetal calf serum (FCS). By using flat bottom micro-titration plate, 1×10^5 BHK-21 cells were inoculated in each well having the volume of 300µL GMEM followed by 2 fold serial dilutions of *E. globulus* oil fractions were prepared and plate was incubation at 37°C under 5% CO_2 concentration (Dutra *et al*., 2012). Sterile PBS solution was used for washing micro-titration plate followed by staining with equal volume of 1% crystal violet and 3% formalin solution then placed for overnight air drying as described by (Almutary and Sanderson 2016) with minor modifications. The volume of 50µL DMSO was added in each well and optical density was taken at 570nm by ELISA plate reader. Cell survival percentage (CSP) was calculated as:

$$C\ S\cdot\ \ \ \ \ \ P\epsilon = \frac{O\ \ \ \ D\ \ \ \ o\ T\ \ -O\ \ \ \ D\ \ \ \ o\ N\iota\ \ \ \ C}{O\ \ \ \ D\ \ \ \ o\ P\ \ \ \ C\ \ \ \ -O\ \ \ \ D\ \ \ \ o\ N\iota\ \ \ \ C} \times 100$$

E. globulus n-hexane plus chloroform fraction was processed for GC-MS for essential active components analysis as described by (Kaur *et al*., 2019). For GC-MS, CARBOWAX capillary column along with helium gas as carrier was used. Injector was heated at 260ºC and sample (n-hexane plus chloroform oil fraction)

injected at 1µL per minute rate. Active compounds in test sample were detected by comparison of retention time with standard compound (Kaur *et al*., 2019).

Statistical Analysis: Data obtained from well diffusion and MIC assay was analyzed through one way analysis of variance (ANOVA) followed by post hoc Duncan's multiple range (DMR) test through SPSS Version 20.0.

RESULTS

Confirmation of *E. faecium:* *E. faecium* (n=04) biochemically characterized isolates, procured from IOM, UVAS, Lahore, were confirmed by Polymerase chain reaction followed by nucleotide sequencing. Gene (16S rRNA) was amplified by PCR and 1500 base pair DNA band visualized by gel documentation system post agarose gel electrophoresis. Amplicons sequenced by Sanger di-deoxy sequencing and FASTA files submitted to NCBI post sequence alignment by n-Blast for accession numbers. All sequenced isolates were declared *E. faecium* based upon nucleotide homology and the accession numbers were MW332526.1, MW332527.1, MW332528.1 and MW332529.1 for Pak 8, 9 10 and 11 isolates, respectively. Phylogenetic tree for *E. faecium* constructed using 16S rRNA sequences, neighbor joining algorithm, bootstrap as phylogeny method and 2000 bootstrap replication. In phylogenetic tree, Pakistan *E. faecium* sequences with accession number represented as colored star (Figure 1). Pak 11 was 73% evolutionary related to the L22 but these both organisms distantly related to IGM5-9. Pak 8, 43% evolutionary related to IGM3-8. Pak 9 and 10 *E. faecium* 47% evolutionary related to each other and 20% related to AA1. Pak10 *E. Faecium* isolate is 53% evolutionary related to MLG20-28.

Antibiotic resistance pattern: The isolates of *E. faecium* (n=04) were analyzed by comparing zone of inhibitions with standard inhibition zones according to clinical laboratory standard institute (CLSI). The highest mean inhibition zone against tested *E. faecium* was of ampicillin (14.00±0.45 mm) followed by oxy-tetracycline (12.11±0.00 mm), amoxicillin (12.00±1.2 mm) and piperacillin (11.35±1.0 mm) while least zone of inhibition was observed in case of norfloxacin (02.00±0.00 mm). Statistically, significant differences were recorded among the various antibiotics tested against *E. faecium* and exhibited variable level of resistance to all of the tested antibiotics as compared to standards. Highest level of resistance against *E. faecium* was recorded in case of erythromycin, norfloxacin and ciprofloxacin which differed non-significantly with each other (Table 1).

Activity of essential oils against antibiotic resistant *E. faecium*: Antibacterial activity of plant essential oils (n=05) was checked against selected *E. faecium* isolates (n=03), which were resistant to multiple antibiotics, by well diffusion test and zone of inhibitions were measured. All of the essential oils exhibited antibacterial activity. Highest mean zone of inhibition was observed in case of *E. globulus* (13.00±1.3 mm) followed by *E. cardamomum* (10.00±1.35 mm), *A. sativum* (06.01±0.03 mm), *C. cyminum* (05.00±1.00 mm) and *F. assa-foetida* (05.00±1.32 mm). Statistically, activity of *E. globulus* was highest against *E. faecium* which differed non-significantly with *E. cardamomum* and significantly with other tested essential oils.

Activity of *E. globulus* essential oil fractions: *E. globulus* was selected on the basis of higher zone of inhibition and nine fractions including n-hexane, n-hexane + chloroform, chloroform, chloroform + ethyl acetate, ethyl acetate, ethyl acetate + methanol, methanol, methanol + acetonitrile and acetonitrile were tested for activity against *E. faecium* antibiotic resistant isolates. Highest mean zone of inhibition recorded was of n-hexane (20.74±0.68 mm) followed by ethyl acetate (15.74±0.50 mm), n-hexane + chloroform (13.45±1.11 mm), chloroform (10.55±0.01 mm), ethyl acetate + methanol (9.33±1.00 mm), methanol + acetonitrile (3.74±1.77 mm) and least for acetonitrile and methanol (0.00±0.00 mm). The solvents used in fractionation were also tested for antibacterial activity on *E. faecium* isolates and no zone of inhibition was recorded. Statistically, n-hexane fraction exhibited higher mean zone of inhibition which differed significantly with rest of the tested solvent fractions against *E. faecium* isolates.

MIC of *E. globulus* fractions: Solvent fractions of *E. globulus* with higher inhibition zones including n-hexane, n-hexane plus chloroform and ethyl acetate were used to determine MIC by micro broth dilution method. MIC value recorded in case of n-hexane plus chloroform (25.28±7.41 mg/mL) was lowest followed by n-hexane (28.13±3.88 mg/mL) and highest for ethyl acetate (50.99±4.38 mg/mL) fraction (Table 2). Statistically mean MIC of n-hexane plus chloroform fraction was lowest which differed non-significantly with n-hexane fraction and significantly with ethyl acetate fraction.

Cytotoxicity analysis of *E. globulus* essential oil fractions: Cytotoxicity analysis was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay to evaluate the safety of mean MIC values of selected plant essential oil solvent fractions and effective concentration 50 calculated in each fraction was higher than the MIC value. In case of *E. globulus* selected solvent fractions including n-hexanes, n-hexane plus chloroform and ethyl acetate cell survival percentages were 52.00, 58.60 and 54.50 at 56.05, 112.10 and 56.05 mg/mL, respectively. Upon comparison with MIC values

of fractions against *E. faecium* isolates EC50 concentrations of *E. globulus* were safe.

GCMS analysis of *E. globulus* essential oil fractions: Least MIC value recorded against *E. faecium* was of *E. globulus* n-hexane plus chloroform fraction which was processed by GCMS analysis for chemical profile. GCMS chromatograms and detailed fatty acid analysis were presented in Figure 2 and Table 3, respectively. Highest percentage in n-hexane plus chloroform fraction was of Bicyclo [2.2.1] heptan-2-ol,1,7,7-trimethyl (14.4%) followed by cyclohexane (13%), benzene (9.5%), Bicyclo[2.2.1]heptan-2-ol (8.7%), cymene (6.4%) and Eucalytptol (5.71%). There were many minor proportions of other chemicals as well.

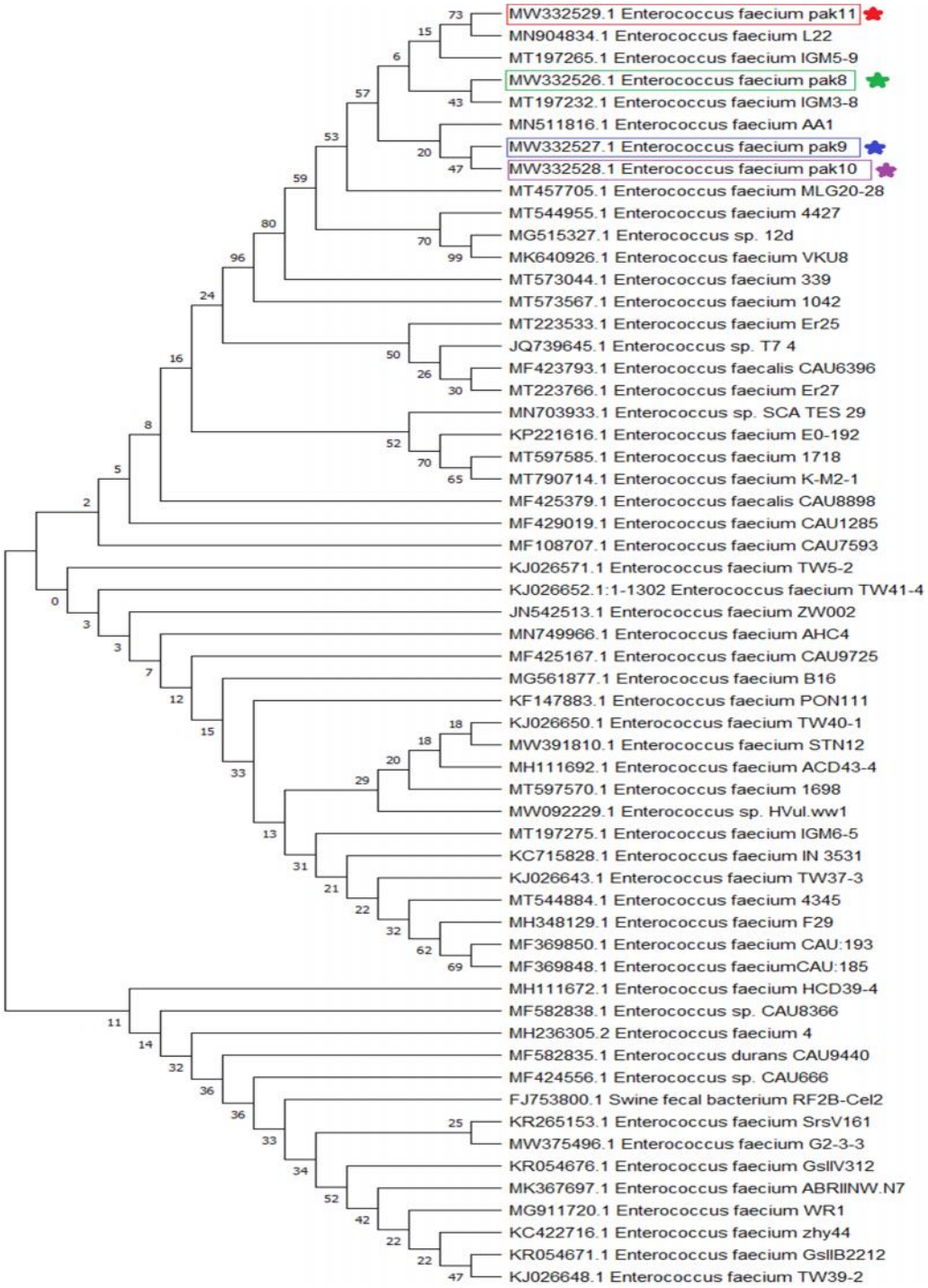

Figure 1: Phylogenetic analysis of *Enterococcus faecium* isolates

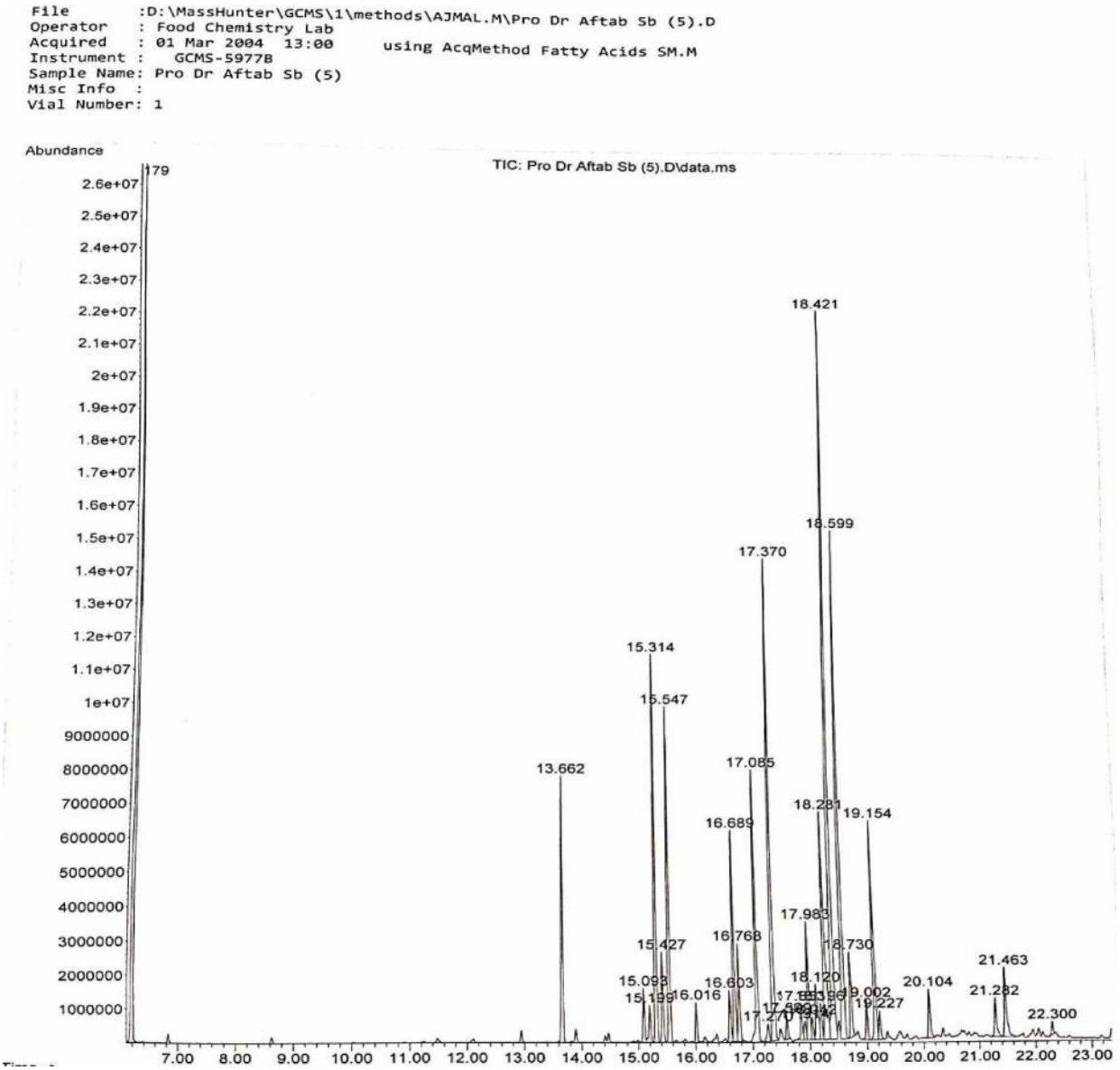

Figure 2: GCMS chromatogram of n-hexane plus chloroform fraction of *Eucalyptus globulus*

Table 1: Mean zone of inhibitions of antibiotic panel against *Enterococcus faecium* isolates (n=04).

Sr. No.	Antibiotic class	Name of Antibiotic	Disc Concentration	Mean test ZOI (mm)	Standard ZOI (mm)		
					S*	I*	R*
01	Penicillin	Ampicillin	AMP 10µg	14.00±0.45^{bc}	≥ 17	-	≤16
		Amoxicillin	AX 25µg	12.00±1.2^{bc}	≥ 17	-	≤16
		Piperacillin	TZP 10µg	11.35±1.0^{bc}	≥ 17	-	≤16
02	Macrolides	Erythromycin	E 15µg	03.01±0.03^{a}	≥ 23	14-22	≤ 13
		Doxycycline	DOX 30µg	09.45±1.22^{b}	≥ 16	13-15	≤ 12
		Oxytetracycline	OTC 30µg	12.11±0.00^{bc}	≥ 19	15-18	≤ 14
03	Vancomycin	Vancomycin	VA 5µg	7.24±1.02^{b}	≥ 17	15-16	≤ 14
04	Fluor quinolones	Norfloxacin	NOR 10µg	02.00±0.00^{a}	≥ 17	13-16	≤ 12
		Ciprofloxacin	CIP 5µg	03.00±0.00^{a}	≥ 21	16-20	≤ 15
		Levofloxacin	LEV 5µg	10.22±2.11^{b}	≥ 17	14-16	≤ 13

^{abc} Means with same super scripts differ non-significantly and with different superscripts differ significantly.
S= Sensitive; I= Intermediate; R= Resistance

Table 2: Minimum inhibitory concentrations of *Eucalyptus globulus* of selected solvent fractions against antibiotic resistant *Enterococcus faecalis* (n=3).

Sr. No.	Solvent fraction	MIC values	Mean MIC values
01	n-hexane	32.00	28.13±3.88[a]
		28. 16	
		24.24	
02	n-hexane plus chloroform	29.56	25.28±7.41[a]
		29.56	
		16.72	
03	Ethyl acetate	56.05	50.99±4.38[b]
		48.46	
		48.46	

[ab] Means with same super scripts differ non-significantly and with different superscripts differ significantly.

Table 3: Chemical profile of n-hexane plus chloroform *Eucalyptus globulus* fraction by GCMS analysis.

S. No.	Type of chemical	RT	Percentage
01	Cyclohexane	6.1	13
02	Bicyclo[2.2.1]heptane, 2,2-dimethyl-3-methylene-	13.6	4.5
03	7-Oxabicyclo[2.2.1]heptane	15	0.87
04	1,3,5-Cycloheptatriene	15.1	0.70
05	o-Cymene	15.3	6.4
06	Cyclohexano	15.4	1.48
07	Eucalyptol	15.54	5.71
08	γ –Terpinene	16	0.62
09	Benzene	16.68	3.6
10	Bicyclo[2.2.1]heptane	16.76	1.7
11	Bicyclo[2.2.1]heptan-2-one	17.0	4.9
12	Bicyclo[2.2.1]heptan-2-ol	17.3	8.7
13	Exo-2,7,7-trimethylbicyclo[2.2.1]heptan-2-ol	17.8	0.62
14	Camphor	18	0.39
15	Isoborneol	18.28	5.35
16	Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl	18.42	14.4
17	Benzene	18.59	9.5
18	L-α-Terpineol	18.7	1.5
19	Acetaldehye	19	0.72
20	Bicyclo[3.1.0]hexane	19.15	3.9
21	Cinnamaldehyde	20.1	1
22	Octanal	21.2	0.9
23	[1,1'-Bicyclopropyl]-2-octanoic acid	21.4	1.7

DISCUSSION

Enterococcus is recognized as indicator organism by US Environmental Protection Agency (USEPA) for bacteriological water quality of fresh and saline water (Harwood *et al*., 2004). *E. faecium* is an opportunistic pathogenic organisms. Ribosomal RNA 16S gene sequence based genotypic identification emerged as accurate, objective and reliable method for bacterial identification with defining taxonomic relationships among bacteria (Clarridge 2004). In present study, *E. feacium* isolates were identified on the basis of 16S rRNA gene sequence analysis on NCBI (nblast). The phylogenetic analysis of the 16S gene of the present four *E. feacium* isolates were used with the selected 54 published sequences of *E. faecium* isolated from different species including human discovered the close exclusion in distinctive clade with 16S gene of *E. faecium* from India and china, respectively. Sequence exclusion pattern discovered random grouping of sequences into different clusters irrespective of species of origin of *E. faecium*. Sequences analysis revealed identity range from 85-100%. Ribosomal RNA 16S sequencing analysis resolves the problem of false negative results for both biochemical and PCR identification (Harwood *et al*., 2004). On the basis of 16S rRNA sequence several species group (phylogenetic) identified in *Enterococcus*

genus (Williams *et al.,* 1991). Similar to above study, from fecal and water samples culturable and nonculturable enterococcus species were identified on the basis of 16S rRNA sequence analysis and most species were *E. faecium* (Ryu *et al*., 2013). In agreement to present study, the Alsanie and colleagues identified MDR enterococcus on the basis of 16S rRNA sequence analysis with 76-100% identity and phylogenetic tree analysis. The sequence variation observed can be endorsed to resistance to antibiotics and gene transfer among the bacterial strains in hospital environment (Alsanie *et al*., 2018).

Enterococcus are not considered as safe and their presences is indicator of fecal contamination (Murray 1990). *E. faecium* associated infections has been increasing and this bacterium found to be more resistant to penicillin and aminoglycosides (Sood *et al*., 2008). A study by Vignaroli and his colleagues reported eight *E. faecium* MDR strains and were resistant to erythromycin, tetracycline and vancomycin (Vignaroli *et al*., 2011). Unal and colleagues observed similar results to Vignaroli and team as 54.9% of *E. faecium* isolates were resistant to three or more antibiotics (Ünal *et al*., 2017). Zheng and team observed similar result of antibiotic resistance as in present study, that *E. feacalis* isolates displayed resistance to erythromycin, tetracycline and vancomycin (Zheng *et al*., 2009). Vancomycin resistant enterococci screening resulted in 1.1% prevalence rate along with 20.7% high level aminoglycosides resistance (HLAR) rate. In Nigerian hospital, among vancomycine resistant enterococci (VRE), prevalent species was *E. faecium* (Shettima and Iregbu 2019). In present study *E. faecium* exhibited variable level of resistance to all of the tested antibiotics. Highest level of *E. faecium* resistance was recorded in case of erythromycin, norfloxacillin and ciprofloxacin.

In case of *enterococci* infections, non-antibiotic therapeutic options are vaccines, nutraceuticals, immune-modulating agents, probiotics, phytochemicals and bacteriophages. Vaccines have been developed to prevent urinary tract infections (UTI) to immune the host against infection containing O antigens, fimbrial subunits, α-hemolysin and siderophores etc. Nutraceuticals are pharmaceutical alternatives that provide health benefits in addition to their basic nutritional value and can be used as medicine. Cranberry (*Vaccinium macrocarpon*), hyaluronic acid, D-mannose, galabiose, vitamin C, chinese herbal medicine (CHM) and phytochemicals including plants and their secondary metabolic derivatives are the nutraceuticals which are used in case of UTI (Loubet *et al*., 2020). Essential oils extracted from star anise, basil, origanum, clary sage and thymus are effective against the multidrug-resistant strains of *Enterococcus* spp. (Ebani *et al*., 2018). Against *E. faecium, E. globulus* essential oil n hexane and n-hexane plus chloroform fractions were proved promising with lowest MIC value with least cytotoxicity.

Essential oils are extensively used in pharmaceutical, cosmetics and in beverage industry and these known as natural plant products (Dosoky and Setzer 2018). Essential oils are used against microorganism to treat different infections. Prior to their in vivo use, thorough in vitro evaluation is very necessary. Cytotoxicity assays was performed for this purpose on several types of eukaryotic cell lines. It was deduced that cytotoxic effect of essential oils and their fractions depends on concentration. The increased concentration of essential oil and fractions increased the cytotoxicity. E. globulus cytotoxicity was ascertained by shrimp lethality testing (Akolade et al., 2012) and results were observed in agreement to Behbahani et al., (2019) which describes as with increased concentration of essential oil fractions, increased cytotoxic effect was observed. The MIC of E. globulus essential oil was used for cytotoxicity by MTT assay and it was revealed that cell survival increased (94-100%) with the decrease of oil concentration.

Eucalyptus species essential oils are used in folk medicines as, analgesic, anti-inflammatory and antipyretic remedies. *E. globulus* fresh leave oil was analyzed through GC/MS and twenty components were identified. Highest percentage (48.6%) was of 1,8-cineole as active component (Daroui-Mokaddem *et al*., 2010). Nearly 45 components from *E. globulus* fruit oil were identified through GLC/MS. Aromadendrene was found in higher percentage (31.17%) followed by 1,8-cineole 14.55% and Globulol 10.69% (Mulyaningsih *et al.,*2010) . Whereas, Compound 1,8-Cineole was found 77.02% in essential oil as 1st active chemical in agreement to Daroui-Mokaddem *et al*., (2010). In present study *E. globulus* essential oil n-hexane plus chloroform fraction was subjected to GC/MS analysis and Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl was found abundant (14.4%) as active compound followed by Cyclohexane (13%) as 2nd active component in essential oil fraction. These findings were in contrast to previous studies.

In conclusion, *E. globulus* oil and its n-hexane plus chloroform fraction may be effective against multi-drug resistant *E. faecium* infections and can be used *in-vivo* because of its lower cell cytotoxicity potential.

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