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GC-MS analysis of yellow pigmented *Macrococcus equipercicus* isolated from alfalfa rhizosphere soil fields of Coimbatore

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Abstract
The rhizosphere of plant possesses important microflora, which secretes wide chemical compounds including secondary metabolites necessary for plant growth and development. The microbial flora of alfalfa plant rhizosphere soil region was explored for functional activity and we found upto ten different pigmented colonies. Due to good functional diversity, this yellow pigmented colony was taken for further studies. Thus, the culture was molecularly characterized and identified for potent bioactive components responsible for antimicrobial activity. The selected culture mass was cultured and secondary metabolites were produced and extracted using ethyl acetate and subjected to GC-MS analysis. The antimicrobial study revealed selective activity against *Streptococcus pneumonia, and Proteus sp* with zone of inhibition to be 18 and 20 mm respectively. Molecular identification of the isolate by 16S rRNA sequencing showed the isolate as *Macrococcus equipercicus* with 100 % similarity. Based on GC-MS analysis report 25bioactive compounds were identified and 13-docosenamide, hexadecanoic acid esters and quercetin were found in ethyl acetate extract. Conclusion: Thus the yellow pigmented gram positive cocci *M.equipercicus* isolated from *Medicago sativa* possessed wide antibacterial activity due to presence of quercetin. Through the studies, we were able to identify potent antibacterial compound producing bacteria from *M. sativa* plant rhizosphere soil.

Keywords: Alfalfa plant, gas chromatography-mass spectrometry, *Macrococcus equipercicus*, *Medicago sativa*, Rhizosphere

INTRODUCTION

Alfalfa plant also known as *Medicago sativa* possesses deep root system andlives in symbiotic association with microbes. The plant has high phy-

to pharmacological importance due to the variety of compounds that has been isolated and identified to cure a variety of diseases (atherosclerosis, heart disease, stroke, cancer, diabetes) (Zhang *et al*., 2006; Bora and Anupam, 2011; Krakowska *et al*., 2017). They are also cultivated worldwide for high protein and fiber for cows. *Macrococcus* sp belongs to family Staphylococcaceae are gram positive cocci, non-motile and non-spore forming (Becker *et al*., 2014).

Studies conducted in our lab showed that alfalfa plant rhizosphere soil contains *Bacillus horikoshii* (Nisha *et al*., 2019a) and *Pantoeaagglomerans* (Nisha *et al*., 2019b) with wide antibacterial activities. *Macrococcusluteus* and *Neisseria sicca* has been reported to be isolated from soil of *Calotropisprocera* and *Catharanthusroseus.* Their extracts possessed antibacterial activities against pathogens (Arora *et al*., 2013). Upon preliminary screening of microbes isolated from rhizosphere soil region of alfalfa plant, this isolate was selected for its amylase, cellulase, protease and phosphate solubilization activities. Therefore, the study was aimed to study the antibacterial activity, culture, extract and identify bioactive compounds of functionally diverse organism from rhizosphere soil region of Alfalfa plant (*M. sativa*) through GC-MS.

MATERIALS AND METHODS

Sample collection: The rhizosphere region soil samples of Alfalfa (*Medicago sativa*) plant were collected from June 2016 to March 2017 at Sulur, Coimbatore, Tamilnadu, India.

Isolation and antibacterial activity: Spread plate method was employed for isolation of microbes from the rhizosphere soil by taking one gram of the collected soil samplesfor serial dilution. About 0.1ml of serially diluted samples (10^{-1} to 10^{-7}) was spreaded onto sterile plates and incubated at 37˚C for 24-72 hours. The isolated colonies were selected and stored in glycerol stocks until further required. The colonies were characterized by staining and biochemical standard methods. The selected isolates were studied for their antimicrobial activities against bacterial pathogens by well diffusion method (Nisha *et al*., 2019a).

Production and extraction of the bioactive compounds: The bioactive compounds were extracted from the culture by large scale cultivation of microbes. After growth, the cells are separated by centrifugation and the supernatant is taken for for extraction process. Ethyl acetate was chosen as solvent for solvent to extract the compounds. After extraction, the solvent were evaporated to collect residues and stored and studied for GC-MS (Nisha *et al*., 2019b).

GC-MS analysis: The microbial extract was further subjected to GC-MS analysis using Thermo MS DSQ II packed DB 35- MS Capillary standard non- polar column. Further the isolated compounds mass spectrums were interpretated by known compounds database NIST.

Molecular identification and phylogenetic analysis: The bacterial genomic DNA isolation were carried out using standard cold spring harbour lab protocol. From the isolated genomic DNA, rRNA genes (1.4 kb length gene)were amplified using the 8 F and 1541 R universal eubacterial primers (5`-AGAGTTTGATCCTGGCTCAG-3` and 5`-AAGGAGGTGATCCAGCCGCA -3` as forward and reverse primers). After amplification, the PCR products were sequenced by big dye terminator cycle sequencing kit (Applied BioSystems, USA) and resolved using Applied Biosystems model 3730XL automated DNA sequencing system (Xcelris Laboratories, India). The phylogeny analyses with multiple closely related sequences were done using MUSCLE 3.7 and PhyML 3.0 aLRT (Nisha *et al*., 2019a and b).

RESULTS AND DISCUSSION

Antibacterial activity: The plant usually releases a variety of carbon and other important nutrients necessary for microbial growth, which makes the mutualistic relationship between plants and microbes at rhizosphere interface (Bertin *et al*., 2003). The microbial extract of yellow pigmented colony studied for antibacterial activity against 7 clinical pathogens namely *P.aeruginosa, Klebsiella sp, S. aureus, Proteus vulgaricus, S. pneumonia, E. coli, B. cereus*revealed that significant zone of inhibition was against against *Streptococcus pneumonia and Proteus sp* (18 and 20 mmrespectively) (Fig. 1). Similar to our study results, Janani *et al*., (2014) studied the pigmented marine bacteria *Exiguobacterium* sp. showing best antimicrobial and antioxidant activities isolated from different regions of India. The *Exiguobacterium* sp. showed activity against *Shigella, Klebsiella*sp and *Staphylococcus aureus*. Similarly studies by Nisha *et al*., (2019a, b) has isolated and reported potential isolates with wide antibacterial activity namely *Bacillus horikoshii* and *P.agglomerans* from alfalfa plant rhizosphere regions.

Molecular characterization of the isolates: The study results of BLAST showed100 % similarity with *Macrococcus equipercicus* (Fig. 2 and 3) and the gene sequences were submitted to the Gene bank (NCBI, USA) and Genebank ID accession number MK240540 received.

GC-MS Analysis: About twenty five compounds were identified from *Macrococcus equipercicus* extract by using GC-MS (Fig. 4). Table 1 and 2 reveal the compounds molecular formula, weight, structure, mass spectra and compound nature and its activities.

The highest intensity (29.58) with retention time of 36.20 showed 13-Docosenamide compound, has

Table 1. *Showing GCMS analysis of compounds obtained from Macrococcus equipercicus extract.*

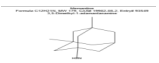
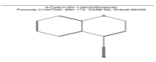
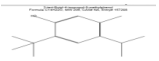
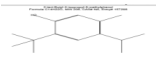
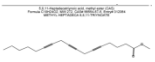
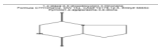
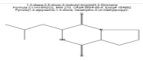
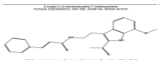
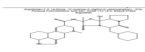
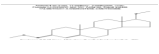
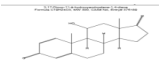
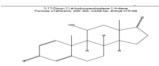
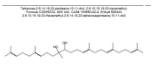
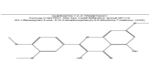
S.N.	RT	Name of the compounds	Molecular formula	Molecular weight	Peak area	Compound structure
1	5.57	Benzene 1,3,5-trimethyl	C_9H_{12}	120	2.71	
2	8.16	Dodecane	$C_{12}H_{26}$	170	3.30	
3	9.18	Memantine	$C_{12}H_{21}N$	179	4.42	
4	10.40	4-Cyano-2H-1-benzothiopyran	$C_{10}H_7NS$	173	5.22	
5	11.29	Tetradecane	$C_{14}H_{30}$	198	2.97	
6	13.25	2-tert-Butyl-4-isopropyl-5-methylphenol	$C_{14}H_{22}O$	206	2.36	
7	13.70	2-tert-Butyl-4-isopropyl-5-methylphenol	$C_{14}H_{22}O$	206	2.36	
8	15.49	Hexadecane	$C_{16}H_{34}$	226	2.23	
9	15.72	5,8,11-Heptadecatriynoic acid,methyl ester	$C_{18}H_{24}O_2$	272	1.65	
10	19.14	1,4-dioxobicyclononane	$C_7H_{10}N_2O_2$	154	2.17	
11	19.59	1-Hexadecanol	$C_{16}H_{34}O$	242	2.05	
12	22.44	1,4-diaza-2,5-dioxo-3-isobutyl bicyclononane	$C_{11}H_{18}N_2O_2$	210	4.41	
13	23.56	Hexadecanoicacid,ethyl ester	$C_{18}H_{36}O_2$	284	4.47	
14	23.68	Hexadecanoic acid , ethyl ester	$C_{18}H_{36}O_2$	284	4.47	
15	26.07	2-Acetyl-3-ethyl-7-methoxyindole	$C_{22}H_{22}N_2O_3$	362	0.95	
16	27.35	Octadecanoicacid,ethyl ester	$C_{20}H_{40}O_2$	312	3.15	
17	27.47	Octadecanoic acid ,ethyl ester	$C_{20}H_{40}O_2$	312	3.15	
18	29.57	Ergotaman-3,6,18-trione	$C_{33}H_{35}N_5O_5$	581	2.83	
19	30.12	Androst-4-en-3-one,17-methoxy,3-methoxime	$C_{21}H_{33}NO_2$	331	3.90	
20	31.76	Lucenin2	$C_{27}H_{30}O_{16}$	610	0.63	
21	32.45	3,17-Dioxo-11-a-hydroxyandrostane-1,4-diene	$C_{19}H_{24}O_3$	300	1.52	
22	33.92	3,17-Dioxo-11-a-hydroxyandrostane-1,4-diene	$C_{19}H_{24}O_3$	300	1.52	
23	36.20	13-Docosenamide	$C_{22}H_{43}NO$	337	29.58	
24	37.34	Tetracosa-2,6,14,18,22-pentaene-10,11-diol,2,6,10,15,19,23-hexamethyl	$C_{30}H_{52}O_2$	444	1.35	
25	37.72	QUERCETIN7,3,4-TRIMETHOXY	$C_{18}H_{16}O_7$	344	0.73	

Table 2. *Activity of compounds identified in Macrococcus equipercicus extract.*

S.N.	RT	Name of the compound	Compound nature	Activity
1	5.57	Benzene 1,3,5-trimethyl	Aromatic hydrocarbon	precursor to styrene
2	8.16	Dodecane	Alkane hydrocarbon	Solvent
3	9.18	Memantine	Amantidine	Treat Alzimer's disease
4	10.40	4-Cyano-2H—benzothiopyran	Aromatic compound	Used in drugs
5	11.29	Tetradecane	Alkane hydrocarbon	Petroleum spirit
6	13.25	2-tert-Butyl-4-isopropyl-5-metylphenol	Lipophilic organic compound	Food additive
7	13.70	2-tert-Butyl-4-isopropyl-5-metylphenol	Lipophilic organic compound	Food additive
8	15.49	Hexadecane	Alkane hydrocarbon	Fuel mixture
9	15.72	5,8,11,Heptadecatriynoic acid methyl ester	Acid compounds	Explosive properties
10	19.14	1,4-dioxobicycloninane	Organic compound	Dehydrohalogenation
11	19.59	1-Hexadecanol	Fatty alcohol	Opacifier
12	22.44	1,4-diaza-2,5-dioxo-3-isobutyl bicyclononane	Not reported	Not reported
13	23.56	Hexadecanoic acid ethyl ester	Saturated fatty acid	antimicrobial, antioxidant, antifungal, 5Alpha reductase inhibitor and hypo-cholesterolemic
14	23.68	Hexadecanoic acid ethyl ester	Saturated fatty acid	antimicrobial, antioxidant, antifungal, 5Alpha reductase inhibitor and hypo-cholesterolemic
15	26.07	2-Acetyl-3-ethyl-7-methyoxyindole	Not reported	Transform Harman alkaloids
16	27.35	Octadecanoic acid ethyl ester	Saturated fatty acid	Confers solubility in organic solvent
17	27.47	Octadecanoic acid ethyl ester	Saturated fatty acid	Confers solubility in organic solvent
18	29.57	Ergotaman-3,6,8-trione	Alkaloid	Inhibits vesicular glutamate transporter activity in cow cerebral synaptic vesi-cles
19	30.12	Androst-4-eb-3-one,17-methoxy,3-methoxime	Aromatic compound	Aromatizing enzyme complex of human placenta
20	31.76	Lucenin2	Glycosyl compound	Not reported
21	32.45	3,17-Dioxo-11-a-hydroxydrstane-1,4-diene	Not reported	Not reported
22	33.92	3,17-Dioxo-11-a-hydroxydrstane-1,4-diene	Not reported	Not reported
23	36.20	13-Docosenamide	Amide of docosenoic acid	Reduces mobility and slightly lessened awareness in cerebrospinal fluid of rat and humam
24	37.34	Tetracosa-2,6,14,18,22-pentaene-10,11-diol,2,6,10,15,19,23-hexamethyl	Not reported	Not reported
25	37.72	QUERCETIN 7,3,4-TRIMETHOXY	Flavanoid	Antioxidant, anthelmintic, antimicrobial, antileishmanial, antiplasmodial

molecular formula is $C_{22}H_{43}NO$ and molecular weight of 337. The compound is amide of do-cosenoic acid, has been reported for *Ludwigia perennis* antimicrobial activity (Sharmila *et al*., 2017). The retention time of 4-Cyano-2H-benzothiopyran from the microbial extract present at 10.40 and its peak area is 5.22, has molecular formula as $C_{10}H_7NS$ and molecular weight is 173 and it is aromatic compound which has the activity in drug usage. The retention time of An-drost-4-en-3-one,17-methoxy,3-methoxime is 30.12 and has peak area is 3.90, has molecular formula is $C_{21}H_{33}NO_2$ and molecular weight of 331 and it is an aromatic compound and it has an aromatizing enzyme complex of human placenta. Quercetin 7,3',4'-trimethyl ether is a trimethoxyfla-vone, derivative of quercetin. The compound

Fig. 1. *Showing antibacterial activity of yellow pigmented colony against Streptococcus pneumonia and Proteus sp.*

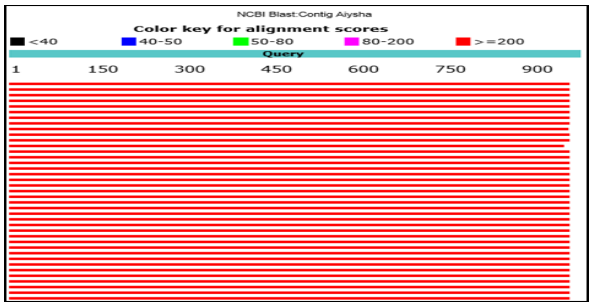


Fig. 2. *Showing multiple alignment scores of Macrococcus equipercicus.*

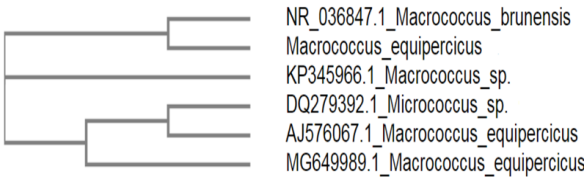


Fig. 3. *Phylogenetic tree of Macrococcusequipercicus based on the 16S rRNA gene sequencing.*

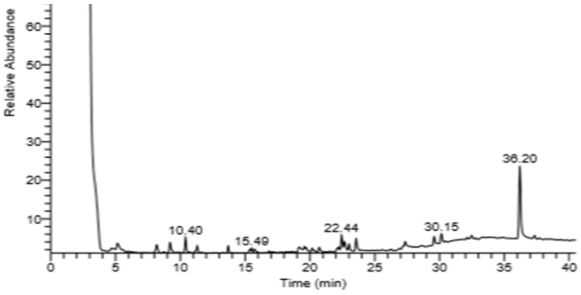


Fig. 4. *GCMS spectrum analysis of Macrococcus equipercicus extract.*

Quercetin 7,3,4-trimethoxy, a flavonoid has been reported for its activities such as antioxidant, anthelmintic, antimicrobial, antileishmanial, antiplasmodial by Kalpana Devi *et al*. (2016).

Conclusion

In present study, the soil isolate from the rhizosphere region of Alfalfa plant of Coimbatore was molecular characterized and identified with 100 % similarity as *Macrococcus equipercicus.* Highest activity was recorded against two pathogens *Streptococcus pneumonia and Proteus sp* which can be due to presence of 13-docosenamide at retention time of 36.2minutes. *In vitro* and *In vivo* biological studies are further necessary to find new drugs against cancer. Through this study we were able to identify potent antimicrobial compounds such as memantine, quercetin and various esters from medicinally important *Medicago sativa.* Thus, the study provides insight into microflora and its bioactive compounds harbouring alfalfa rhizosphere soil region.

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