Original article

In silico identification of novel and conserved microRNAs and targets in peppermint (*Mentha piperita*) using expressed sequence tags (ESTs)

Dhivya Selvaraj [a,*,1], Susithra Priyadarhni Mugunthan [b], Harish Mani Chandra [b,1], Chandramohan Govindasamy [c], Khalid S Al-Numair [c], Ravindran Balasubramani [d]

[a] Department of Computer Science and Engineering (CSE-AI) Amrita School of Engineering, Amrita VishwaVidhyapeetham, Chennai, India
[b] Department of Biotechnology, Thiruvalluvar University, Vellore 632115, Tamil Nadu, India
[c] Department of Community Health Sciences, College of Applied Medical Sciences, King Saud University, P.O. Box 10219, Riyadh 11433, Saudi Arabia
[d] Department of Environmental Energy and Engineering, Kyonggi University, Suwon-si, Gyeonggi-do 166227, Republic of Korea

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ABSTRACT

Objectives: *Mentha piperita,* generally known as peppermint is a popular herb cultivated in the temperate region around the world belongs to the family Lamiaceae. *M. piperita* is widely used as traditional food flavoring, in addition they are as well used to treat cold, fever and throat inflammation, and they have anti-viral and anti-fungal properties. The characteristics of MicroRNAs (miRNAs) includes, small size with single stranded non-coding regions having 22 nucleotides. They are evolutionary conserved and actively involved in post-transcriptional regulation of RNAs, which plays various important roles in metabolic and cellular processes of animals and plants.
Methods: The present study reports the potent computational method based on homology search used to find potential miRNA of peppermint. EST sequences were retrieved from NCBI GenBank and BLAST against with plant miRNAs. By various filtration criteria we identified potential miRNAs from miRNA family with suitable properties.
Results and Conclusion: A total of 1216 ESTs were and 1188 unigenes were identified in this study. A set of prospective target genes were identified in peppermint based on sequence homologies mainly encodes for transcription factor, transport inhibitor like protein, GRR1-like protein and DNA-binding protein. The present study endows valuable information on miRNA in peppermint which will accelerate further research in peppermint.
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1. Introduction:

Mint belongs to the family Lamiaceae a largest family of plant with 236 genera and 7000 species (Marzouk et al., 2018) and genus *Mentha,* consist of 42 species, 15 hybrids and subspecies, cultivars and varieties (Kalemba and Synowiec, 2020). *Mentha piperita* is generally called as peppermint is a perennial herb widely used by greater part of people globally in various forms like leaf, leaf extract oil and leaf water (Ekor, 2014). Due to its rich flavor and fragrance peppermint has economic value both in the domestic and international market and an important export commodity that fetches good foreign revenue. Peppermint is a valuable plant for its distinctive aroma and medicinal value. It is a resource for various chemical substances like terpenoids and phenylpropanoids, having varied chemical structures that are part of particular metabolism (Sangwan et al., 2001). It is also rich in enormous bioactive compounds and contains sugars, saponins, alkaloids, anthraquinones, and quinines (Padmini et al., 2008).The peppermint oil logs for various antispasmodic activities like antivomiting, carminativum, stomachic and antimicrobial properties. The main compounds such as menthol, menthone, and menthofuran which gives unique aroma (da Silva Ramos et al., 2017).

miRNAs are tiny non-coding RNAs, the length is about 22–24 nucleotides in size. A DNA sequence first transcribes to primary miRNAs followed by precursor miRNAs and in turn into mature miRNAs. Mostly, miRNAs associate with the target mRNAs by 3′ UTR to curb its

* Corresponding author.
E-mail address: s_dhivya@ch.amrita.edu (D. Selvaraj).
[1] These authors contributed equally.

expression (Denli et al., 2004). It also interacts with gene promoters and 5′ UTR coding sequence (Broughton et al., 2016); in addition under specific condition it activates gene expression (Vasudevan, 2012). Besides it shuttles among diverse sub-cellular organelles to control the frequency of transcription and translation (Makarova et al., 2016), involves in differentiation and development process, abiotic and biotic stress response (Ren et al., 2012). With robust computational and experimental approaches several miRNAs were identified in plants (Bartel and Bartel, 2003), and through comparative genomics approach these miRNAs were found to be highly conserved among species. This miRNA can also be identified by the use of expressed sequence tag (EST) and a Genome Survey Sequence (GSS) (Zhang et al., 2007). EST analysis has gained significance when compared with other approaches like conserved miRNAs and be capable of identifying without knowing the whole genome sequences. It offers straight confirmation for miRNA expression than from genomic sequence surveys without any much specified software. By this method several miRNAs are effectively recognized in diverse plant species.

The genome-wide miRNA profiling have been developed using three major platforms like small RNA cloning, microarray and qRT-PCR further by miRNA-Seq which provides single-nucleotide resolution of miRNA species. The deep sequencing approach is yet to be successfully developed. It is a challenge to identify unique miRNAs from the big group of small RNA sequences. Hereby *in silico* computational approach is employed in identification of novel and conserved miRNAs in peppermint which has immense medicinal and economic importance. In the current research we had recognized novel and conserved miRNAs using expressed sequence tags (ESTs) of peppermint. miRBase is a public miRNA database that has been first deposited by Kozomara et al., (Kozomara and Griffiths-Jones, 2014). In current research, we applied expressed sequence tag-based homology search to discover miRNAs in peppermint by presently available 1216 expressed sequence tags ESTs from NCBI EST database. From the outcomes of our results, only one possible miRNA, from the peppermint family has been identified. This recently recognized miRNA plays an essential function in diverse biological processes. This study reveals the key features of recognized peppermint miRNA.

2. Methods

2.1. Computational reorganization of mint miRNA

In total 74 mature miRNA taken from miRNA sequence database miRBASE Version 21 (https://www.mirbase.org). To find conserved and novel miRNAs in peppermint, 43 distinctive miRNAs were predicted and chosen for next studies. On the whole 1216 EST sequences of peppermint (*Mentha piperita*) are taken in NCBI dbEST database (https://www.ncbi.nlm.nih.gov/nucest/). Blast 2.2.30 + an offline version of BLAST was used for further studies (ftp://ftp.ncbi.nlm.nih.gov/blast). Nearly 1188 contigs were identified using CD HIT server from which 1061 Unigenes were annotated.
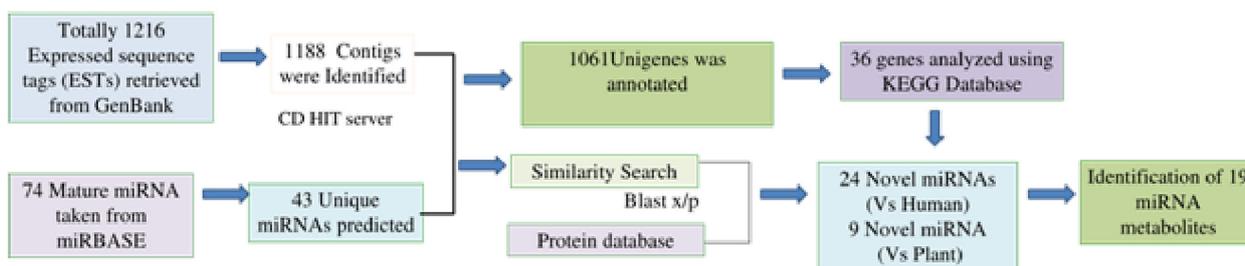
2.2. Expressed sequence tag assembly and functional annotation

The ESTs were assembled employing CLC Genomics Workbench 8.0 (https://www.clcbio.com/products/ clc-genomics-workbench/). Gene ontologies and functional annotation were analyzed by BLAST2GO suite21. BLASTn and BLASTx servers were employed in similarity search against the viridiplantae family. The unigenes were annotated based on similarity search using of e-10.

2.3. Mature miRNA identification

Pair-wise alignment of assembled EST sequences against a unique miRNA database with a threshold value of e-10 and window size of 7 was carried out using the BLAST algorithm using mature miRNAs extracted from the miRBase database. miRNA candidates were selected using homology search approach of the threshold of 18nt length with no gaps. Further study was followed by the known miRNAs were selected that are closely matched with the assembled sequence and subject to BLASTx search against non-redundant protein database. Fig. 1 represents the miRNA scheme and EST analysis in peppermint.

2.4. miRNA secondary structure prediction

The non-protein coding predecessor sequence was employed to envisage the secondary structure using Mfold software which uses energy minimization method, protein coding sequences were removed prior to prediction (Kozomara and Griffiths-Jones, 2014; Zuker, 2003), filter criteria was followed to select pre-miRNA with the length of 60 nt properly folded to form stem-loop-hairpin secondary structure with maximum of 6 nt mismatches. There must be no breaks allowed between the miRNA/miRNA* duplex, A + U content must be between 30 and 70 percent, and the projected secondary structure must have a higher minimal folding free energy index (MFEI) and negative minimal folding free energy (MFE), which distinguish miRNAs from short RNAs. The negative free was calculated using the fact that:

$$\text{MFEI} = \frac{\text{MFE} \times 100}{\text{Length of pre miRNA sequence}}$$

2.5. miRNA target genes prediction

The important criteria for miRNA target prediction are seed match, free energy, conservation, target site abundance and site accessibility. The target miRNAs were predicted using homology algorithm (Rhoades et al., 2002), psRNA target server was used to find miRNA targets with default parameters. The prospective miRNAs as query are searched against with mRNAs and assembled ESTs of peppermint. Target accessibility was granted maximum energy to unpair the target site (UPE) by adhering to the specifications of maximum exception being 3.0, length of complementarily scoring (hspsize) being 20, and flanking length approximately about the size of target site for accessibility analysis which is

Fig. 1. Illustrative representation of the method followed for the identification of miRNAs from mint ESTs.

13 bp downstream and 17 bp upstream. The highest mismatch at the complementary site is 4 without gaps, while the choice of the central mismatch resulting to translational inhibition is 9–11 nucleotides.

2.6. Phylogenetic analysis of mature miRNAs

The standalone blast search tool was used against miRBase database and finally matured miRNAs were predicted. Multiple sequence alignment tool ClustalW, used to align sequence of precursor miRNA and newly identified miRNAs. MEGA version 6.25

Table 1
Summary of ESTs assembly and annotation in *Mentha piperita*.

Item	Number
Total number ESTs	1216
Number of contigs	1188
Total number of bases in unigenes	617,396
Maximum length of contigs	1147
Minimum length of contigs	157
Average length of contigs	519
Number of unigenes annotated	1061
Total number of unannotated unigenes	93

was used to conduct phylogenetic and molecular evolutionary analyses and phylogenetic tree was built using maximum likelihood method (Olsen et al., 1994).

2.7. Functional annotation and pathway analysis of miRNA target genes

The target miRNA gene sequences were matched to the non-redundant protein sequence database of NCBI GO keywords using the BLAST2GO 3.0 tool (https://balst2go.com/blast2go-pro/download-b2g). The Gene Ontology (GO) database was used to look into biological processes, cellular components, and molecular functions. The Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto, 2000) KASS server was used to annotate target miRNAs, its metabolic pathways, and their networks (Moriya et al., 2007).

2.8. Structure prediction of key metabolites and its binding pockets of M. Piperata

The 3D structure and active sites of identified key metabolites like (s) -limone 3- monooxygenase, (+) menthofuran synthase, (+) pulegone reductase, (+) neomenthol dehyrogenase and vestitone reductase were modeled and predicted by RaptorX (https://raptorx.uchicago.edu/BindingSite/) (Källberg et al., 2012).
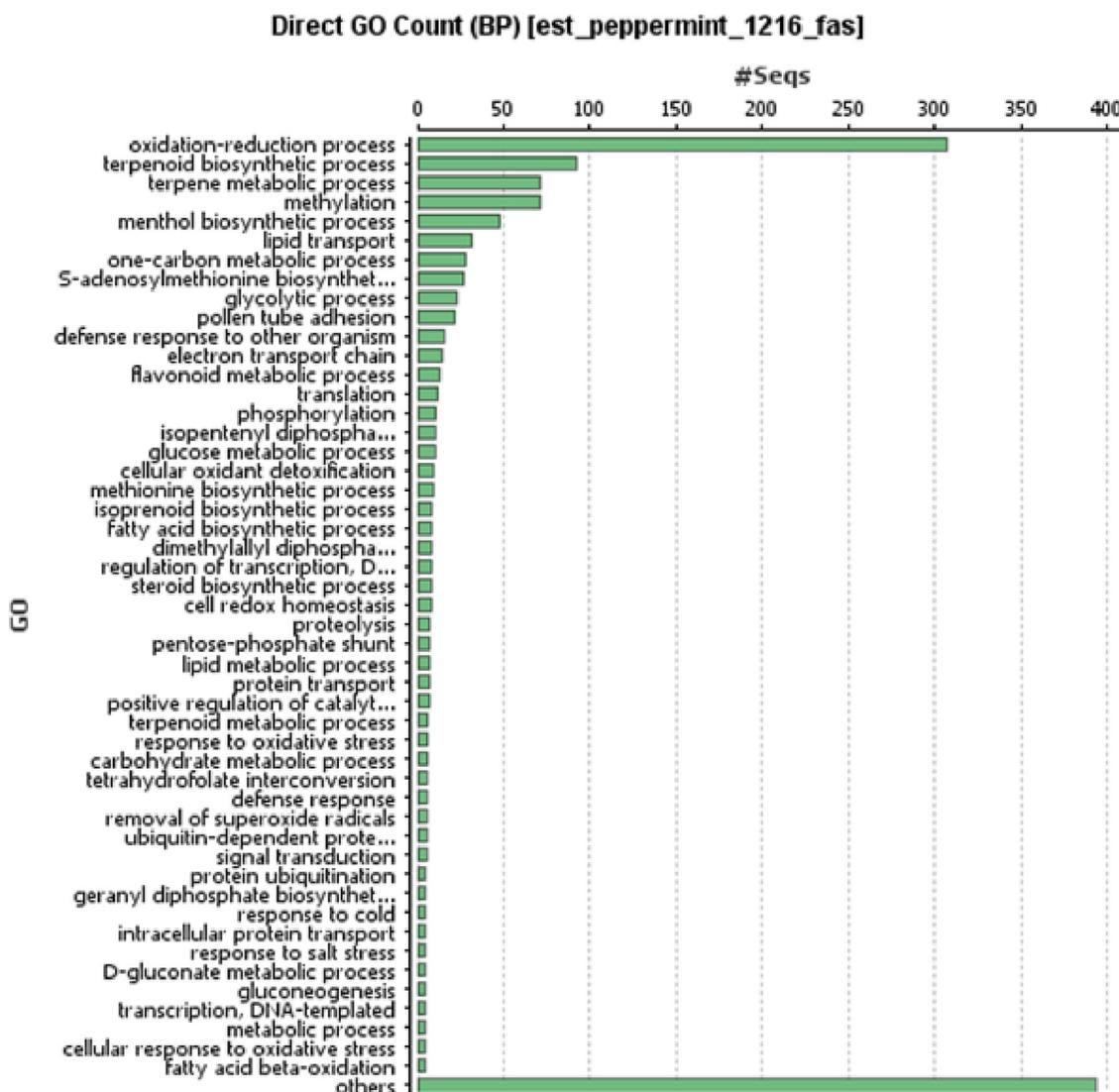

Fig. 2. Species distribution of mint unigenes based on sequences similarity search.

3. Results

The dbEST database yielded a total of 1216 EST sequences, which were subsequently assembled and functionally annotated. 1188 unigenes were produced by the EST assembly after redundancy was eliminated. For peppermint unigenes, the typical read length was 519 bp. Table 1 displays the summary data for EST assembly, clustering, and annotation.
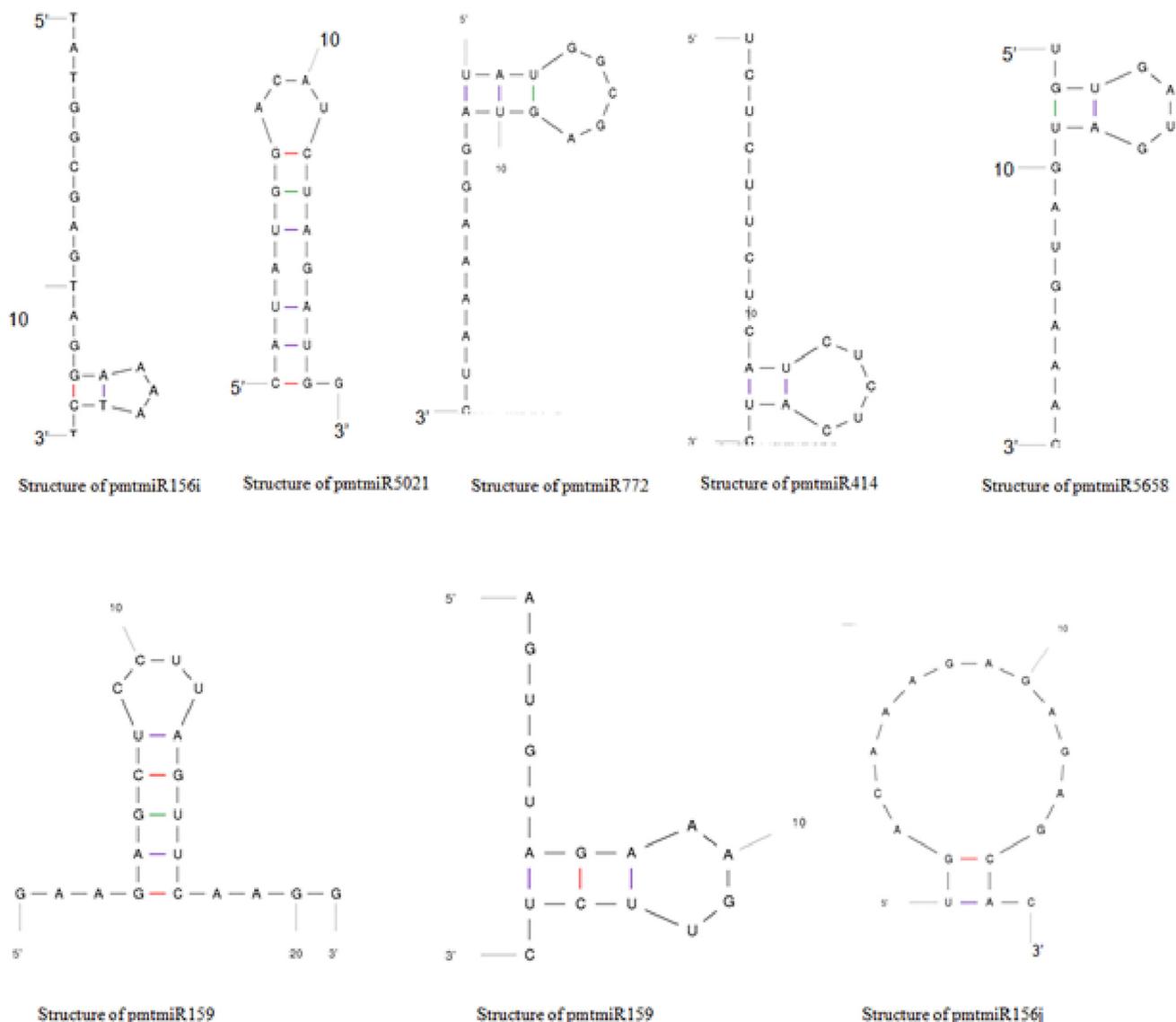
The BLAST2GO suite was used, and unigenes were mapped and assigned to Gene Ontology (GO) keywords of the unigenes shown in Fig. 2 for the similarity search BLASTx against numerous public databases. 1061 (87.25 %) of the 2710 unigenes received significant hits in the similarity search, while 93 (7.6 %) had no significant similarity. One species only, *M. piperita*, is thought to contain unannotated unigenes. Additionally, the M. piperita unigenes demonstrated the utmost likeness with *Musa acuminata*, followed by *Phoenix dactylifera* and *Elaeis guineensis*.

Table 2 shows the Gene Ontology (GO) terms that were assigned to unigenes. Further characterization includes oxidation reduction, terpenoid biosynthesis, terpene metabolism, methyla-

Table 2
Identification of novel miRNA in mint ESTs.

Source	Homologous miRNA	% of AU of Pre-miRNA	Free energy Kcal/ mol	Predicted miRNA sequence	LM	MN	e- value
miRNA-1	pmtmiR159	50	−50.90	UAUGGCGAGUAGGAAAAUCU	20	0	1.9
miRNA-2	pmtmiR5021	58	−53.33	CAUAUGGACAUCUAGAUGG	19	1	1.7
miRNA-3	pmtmiR772	58	−52.78	UAUGGCGAGUAGGAAAAUC	19	2	1.5
miRNA-4	pmtmiR414	58	−67.50	UCUCUUCUCAUCUC UCAUC	19	3	3.7
miRNA-5	pmtmiR5658	64	−63.70	UGUGAUGAUGAUGAAAC	17	2	3.5
miRNA-6	pmtmiR159	52	−65.25	GAAGAGCUCCUU AGUUCAAGG	21	2	3.5
miRNA-7	pmtmiR8166	58	−44.69	AGUGUAGAAAGUUCUC	17	2	0.41
miRNA-8	pmtmiR156j	52	−27.22	UGACAAAG AGAGAGCAC	17	2	0.41
miRNA-9	pmtmiR5021	77	−40.57	UGAGAAGAAGAA AAAA	18	4	3.2

Structure of pmtmiR156i   Structure of pmtmiR5021   Structure of pmtmiR772   Structure of pmtmiR414   Structure of pmtmiR5658

Structure of pmtmiR159   Structure of pmtmiR159   Structure of pmtmiR156j

Fig. 3. Structures of predicted mRNA.

tion, menthol biosynthesis, lipid transport, one-carbon metabolism, and S-aenosylmethionine production, among other molecular processes. Small molecular binding, transferase activity, and hydrolase activity genes all played an important role in molecular functioning. Genes involved in metabolic, single-organism, cellular, and biological processes have been found. Furthermore, the cell's cellular components had the greatest representation in both cell and organelle.

Peppermint unigene sequences have been matched with Kyoto encyclopaedia of genes and genomes reference pathways (KEGG). On the whole, 36 number of KEGG pathways was recognized with biosynthesis of monoterpene as the mainly ample in the classes of enzyme (+)-menthofuran synthase, (+)-pulegone reductase, (4S)-limonene synthase and vesititone reductase, followed by ATP-dependent degradation of ubiquitinated proteins pathways. This study supported the customary idea about peppermint having medicinal values.

3.1. Identification of miRNA in peppermint ESTs

A total of 1188 unigenes were used to identify miRNAs, with the majority of mature plant miRNAs being evolutionarily conserved.

MiRBase (https://www.mirbase.org/cgi-bin/browse.pl) was used to obtain 74 mature miRNAs. After eliminating the duplicated sequences, 43 unique miRNAs were used as a reference, and 1188 unigenes were BLASTed against them using settings such as e-10, word, and window size of 7. With 9 unigenes, the search yielded 24 possible mature miRNA showing homologs. The anticipated miRNA length is between 16 and 22 nucleotides, which is consistent with earlier plant research. Mfold software was used to create secondary structures for selected miRNAs, and various filters were employed to pick candidate miRNAs. Nine miRNAs had higher free energy and content as prospective miRNAs, out of a total of 24. The stability of the hairpin stem loop secondary structure of the precursor miRNA was determined using the terms minimum free energy (MFE) and anticipated value (E-value). Therefore, a lower value of MFE implies that the secondary hairpin stem loop structure of the precursor miRNA is more stable. Fig. 3 provides the anticipated mRNA structures.

This study shows the occurrence of nine miRNA from 1216 ESTs of peppermint, according to Zhang, et al 2007 the likelihood of discovering miRNA is one per 1000 ESTs. The nucleotide percentage for recently recognized miRNA in peppermint ESTs are tabulated in Tables 3 and Table 4.

Table 3
Nucleotide percentage for newly identified miRNA in mint.

miRNA	Mature miRNA sequence	miRNA family	A%	C%	G%	U%	A/U	G/C	A + U%
miRNA-1	UAUGGCGAGUAGGAAAAUCU	pmtmiR5021	35	10	30	25	1.4	2.5	60
miRNA-2	CAUAUGGACAUCUAGAUGG	pmtmiR5021	31.5	15.8	26.3	26.3	1.2	1.6	58.1
miRNA-3	UAUGGCGAGUAGGAAAAUC	pmtmiR772	36.8	10.52	31.5	21	1.75	3	57.8
miRNA-4	UCUCUUCUCAUCUCUCAUC	pmtmiR414	10.5	42.1	0	47.3	0.2	0	57.8
miRNA-5	UGUGAUGAUGAUGAAAC	pmtmiR5658	35.3	5.88	29.4	29.4	1.2	5	64.7
miRNA-6	GAAGAGCUCCUUAGUUCAAGG	pmtmiR159	28.6	19.0	28.6	23.8	1.2	1.5	52.4
miRNA-7	AGUGUAGAAAGUUCUC	pmtmiR8166	31.3	1.25	2.5	31.3	1	0.5	62.6
miRNA-8	UGACAAAG AGAGAGCAC	pmtmiR156j	47	18.7	29.4	5.88	8	1.7	52.8
miRNA-9	UGAGAAGAAGAAAAAA	pmtmiR5021	68.7	0	25	16	11	4	84.7

Table 4
List of potential target genes of mint miRNA.

S.No	Accession No	Target Protein	miRNAs	Inhibition KO number	GO Annotation Biological process) Functions
1	AW254845.1	histone H1-like	ML1267	K11275	Stabilizes chromatin structure
2	AW254953.1	(S)-limonene 3- monooxygenase	ML1122	K15089	Monoterpene biosynthesis
3	AW255209.1	26S protease regulatory subunit 6A	ML201	T03446	ATP-dependent degradation of ubiquitinated proteins
4	AW255784.1	26S protease regulatory subunit 6A	ML852	K03065	ATP-dependent degradation of ubiquitinated proteins
5	AW255882.1	(+)-menthofuran synthase	ML968	K15093	Monoterpene biosynthesis
6	AW255952.1	(+)-pulegone reductase	MW298	T10000	Monoterpene biosynthesis
7	AW255510.1	glycine-rich protein A3-like	ML536	T05027	Interaction in cell wall associated Proteins
8	AW254888.1	(4S)-limonene synthase	ML1312	K15088	Biosynthesis of *sec* metabolite
9	AW255223.1	4-hydroxy-3-methylbut-2-en-1-yl diphosphate reductase	ML218 K03527		Isoprenoid biosynthesis
10	AW255083.1	(-)-camphene/tricyclene synthase, chloroplastic-like	ML1378	K07385	1,8-cineole synthase
11	AW254725.1	(-)-alpha-terpineol synthase- like	ML1011	T01084	Monoterpenoid biosynthesis
12	AW255955.1	(+)-neomenthol dehydrogenase	ML536	K15095	Monoterpenoid biosynthesis
13	AW255293.1	S-denosylmethionine synthase	ML300	K00789	Methylation in response to stress
14	AW255832.1	*trans*-resveratrol di-O-methyltransferase	ML900	K16040	Stilbenoid, diarylheptanoid and gingerol biosynthesis
15	AW25593.1	(+)-pulegone reductase	MW188ED	K15092	Monoterpene Biosynthesis
16	AW255630.1	acyl-coenzyme A thioesterase	ML69	K17362	Activates thiolester hydrolase
17	AW255783.1	vestitone reductase	ML851	K13265	Isoflavonoid biosynthesis
18	AW255061.1	membrane- associated progesterone receptor	ML1356	K17278	Progesterone binding
19	AW255186.1	MADS-box transcription factor	ML174	K09264	Cytoskeletal dynamics

3.2. miRNA target prediction

Peppermint contigs/unigenes were analysed for the miRNAs prediction which regulates gene expression and actively involved in growth development, stress stimulus, defense mechanism. psRNA target server, a homology based tool was employed in prediction of peppermint miRNA from target mRNAs. A total of 19 targets for the new identified mature miRNAs of peppermint were reported. Among 19, nine were of miRNAs which are represented in the Table 3.

3.3. Phylogenetic analysis

The anticipated precursor miRNA sequences were aligned using Clustal W, and phylogenetic analysis was utilized to establish the evolutionary link using MEGA's maximum likelihood technique.

MW298 and ML1312 were discovered to be closely related among 19 predicted miRNA sequences that are active in Monoterpene biosynthesis, indicating (+)-pulegone reductase and (4S)-limonene synthase, respectively, as illustrated in Fig. 4.Fig. 5..

3.3.1. KEGG pathway analysis and GO term annotation

To better comprehend the roles of the newly discovered target genes, Gene Ontology (GO) concepts were applied. In terms of biological process, molecular function and cellular components, the targeted genes were assigned to regulatory networks that are active in oxidation reduction process, terpenoid biosynthesis, terpene metabolism, methylation, menthol biosynthesis, lipid transport, one carbon metabolism, S-adenosylmethionine biosynthesis, glycolytic process, pollen tube adhesion defence response, integrated component membrane, and cytoplasm endoplasmic reticulum.
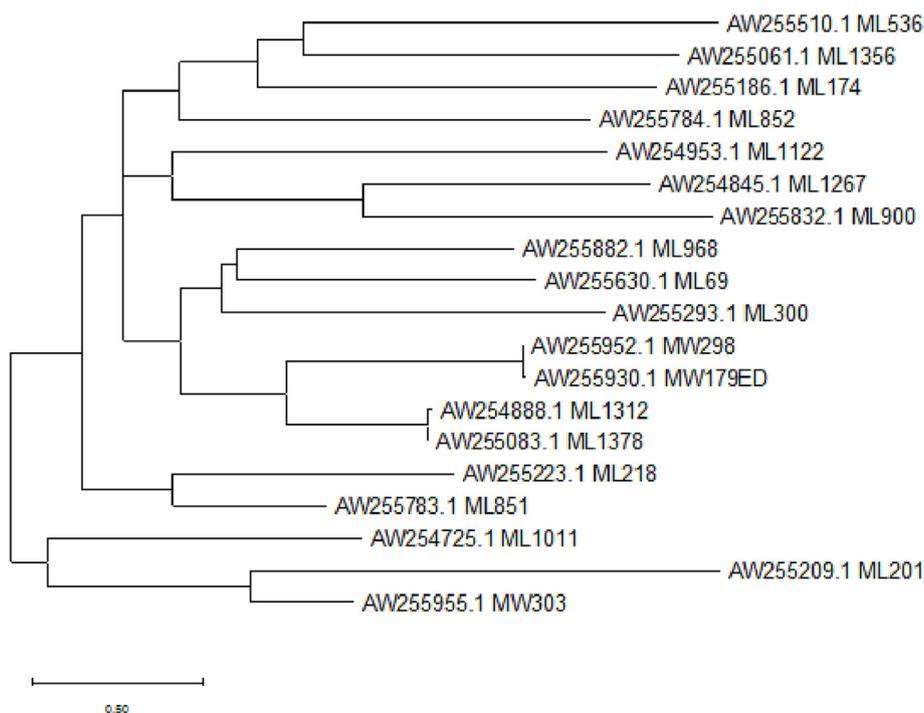

Fig. 4. Phylogenetic tree prepared using maximum likelihood method showing relationship between newly identified pre-miRNAs of mint with their closely related families.
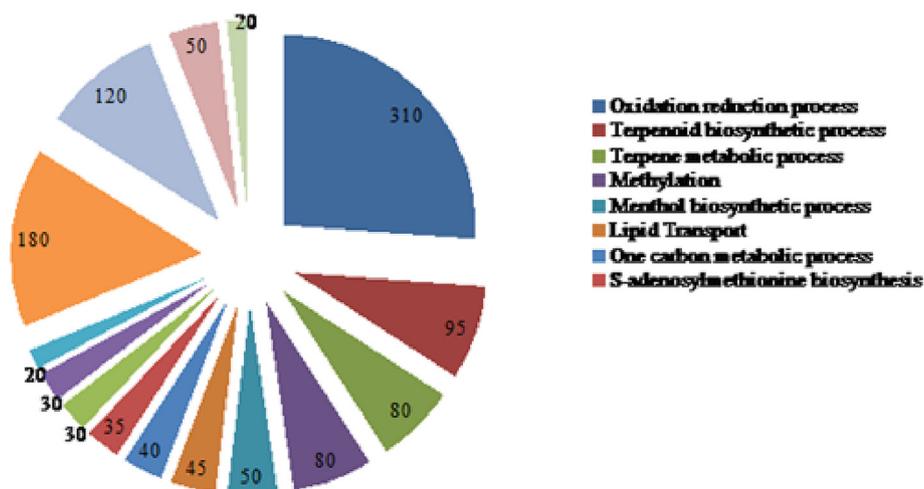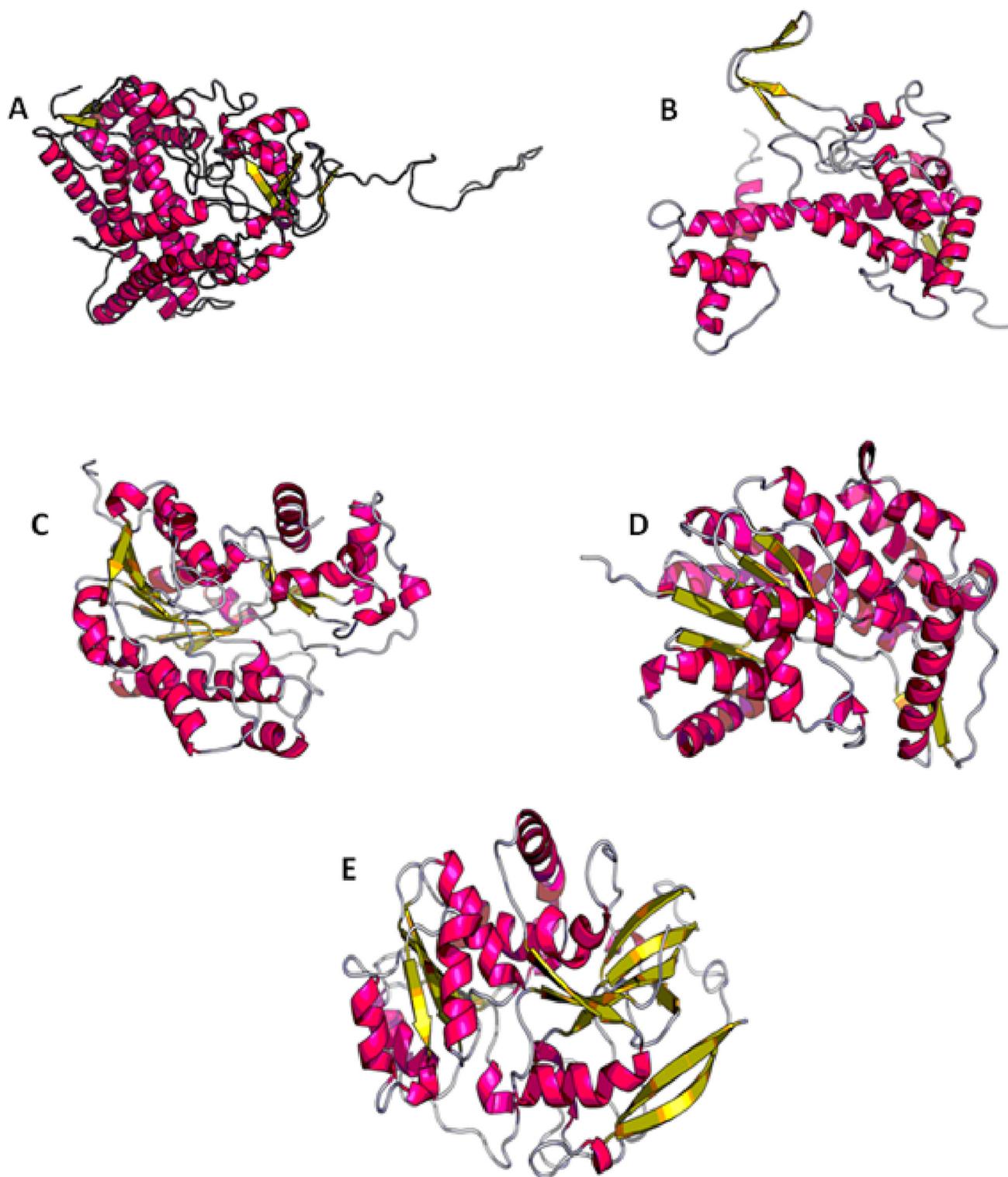

Fig. 5. Functional annotations of miRNAs in *M.piperata*.

Fig. 6. Modelled structure of key metabolites and binding pockets of *M. piperata*. (A) Binding sites of (s) -limone 3- monooxygenase [G12 N14 K15 G16 I17 R37 L65 D66 V67 N93 A94 G95 V96 V180 S181 K242 G267 A269 T271 D272 273 T274]. (B) Binding sites of (+) menthofuran synthase [T68 A71 G72 T75 S76 T79 M130 P135 I136 I139 P140 R141 P201 F202 G203 R207 I208 C209 P210 G211 F214 G215 V219]. (C) binding sites of (+) pulegone reductase [G15 T17 G18 F19 L20 I39 R40 L69 M88 A89 H90 P91 L92 T130 S131 S132 Y168 K172 P195 T196 W197 I198 S210]. (D) binding sites of (+) neomenthol dehyrogenase [R99 V115 A116 R128 M292 A295 G296 T299 T300 A303 P359 F360 L363 L364 R366 P426 F427 G428 R432 G433 C434 P435 G436 F439 A440 Y444]. (E) Binding sites of vestitone reductase [P52 M135 T139 G163 S164 V165 A184 G185 K189 Y205 N229 V230 C251 G252 V254 S255 Y257 F281 V282 V283 V326 G329].

3.4. Structure prediction of key metabolites and its binding pockets of M. Piperata

The binding pocket residues were found in the enzymes (s) - limone 3- monooxygenase (EC:1.14.14.143), (+) menthofuran synthase (EC:1.3.1.81), (+) pulegone reductase (EC:1.3.1.81), (+) neomenthol dehydrogenase (EC:1.3.1.81), and vestitone reductase (EC:1.3.1.81) (EC: 1.1.1.348). a. Binding pocket locations were utilised to assess the quality of an enzyme's predicted structure. It denotes the number of times the selected binding pocket was dis-
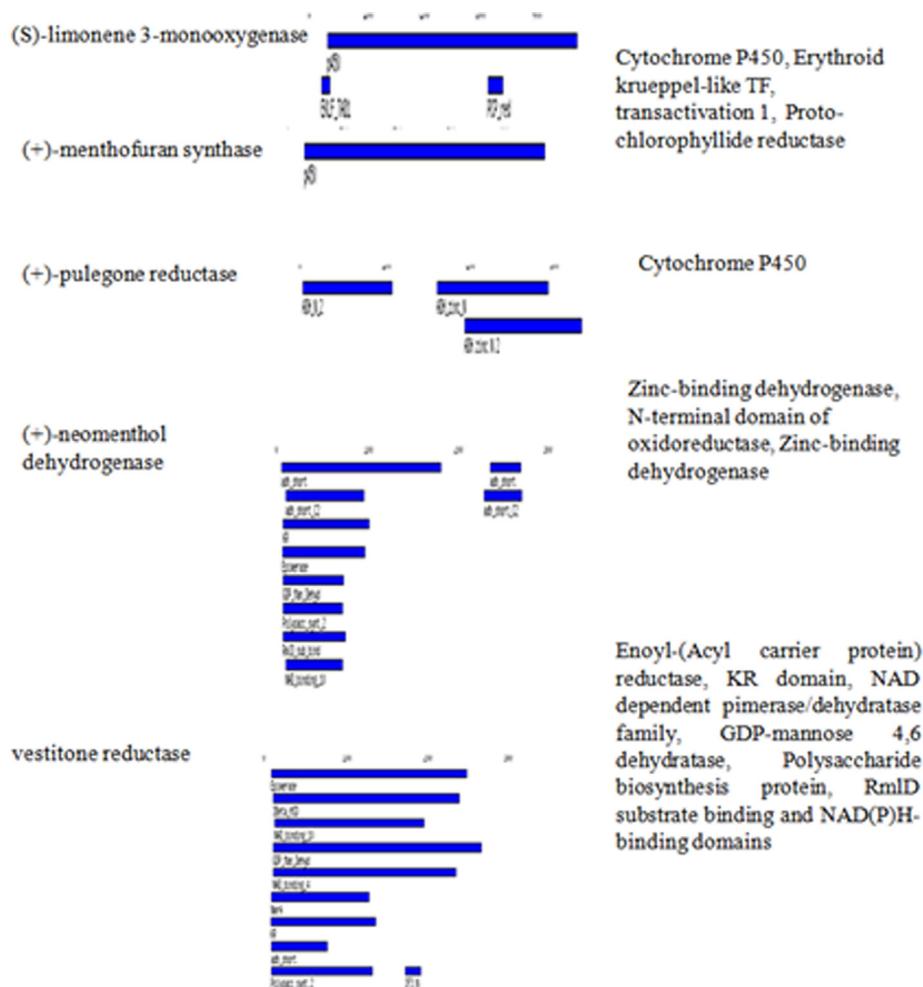
Fig. 7. Target motif metabolites gene in *M. piperata.*

covered in a set of important metabolites. From 19 to 23 residues, the number of binding pockets and their multiplicity were changed. Figs. 6 and 7 shows the binding residues of important metabolite structures as well as their positions. The hydrophobicity of the binding residues is roughly 50%.

4. Conclusion

The peppermint (*Mentha piperita*) is known for its medicinal properties. EST sequence of *M. piperita* was used for miRNA prediction and target identification by computational approach. The present study identified nine novel miRNA and their targeted genes in peppermint that has major significance in metabolic process. The identified 41 target genes modulate a variety of regulatory molecules, including transcription factors, enzymes, and secondary messengers, to control the expression of several genes. By computational approach this study systematically identified the miRNAs and characterizes the genes and their potential regulatory miRNAs for the synthesis of bioactive components such as limonene, menthofuran, pulegone, neomenthol and vestitone of terpene family. Conclusively these finding may further enhance our understanding towards peppermint miRNAs and their importance in biological process.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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