INVESTIGATION OF SOME PHYTOCHEMICAL CONSTITUENTS AND BIOACTIVITIES OF LEAF EXTRACTS OF *MENTHA SPICATA* L. (PU SI NAN)

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Abstract

The present work focused on the investigation of nutritional values, antimicrobial activity, antioxidant activity and some phytoconstituents in the leaf extracts of *Mentha spicata* L. (Pu Si Nan). The preliminary phytochemical tests revealed the presence of alkaloids, α-amino acids, carbohydrates, cyanogenic glycosides, flavonoids, glycosides, organic acids, phenolic compounds, reducing sugars, saponins, steroids, tannins and terpenoids. However, starch was absent. The nutritional values were determined by AOAC method providing proteins 23.68 %, ash 25 %, fibers 11.49 %, water content 15.15 %, carbohydrates 17.04 %, fats 7.64 % and energy value (232 kcal /100 g). *Invitro* screening of antimicrobial activity by agar well diffusion method on five different microorganisms (*Escherichia coli, Enterococcus faecalis, Staphylococcus aureus, Bacillus cereus, Candida albicans*) on PE, EtOAc, EtOH, $CHCl_3$ and H_2O extracts of the leaf of *Mentha spicata* L. (Pu Si Nan), PE, EtOH, $CHCl_3$ and EtOAc extracts showed antimicrobial activity on four strains of microorganisms (except *E. coli*). Watery extract was observed activity against on *E. faecalis*, *S. aureus* and *B. cereus*. The highest inhibition zone was observed 20.50 ± 0.35 mm on *Bacillus cereus* by EtOAc extract of sample. The antioxidant activity of watery and ethanol extracts of the leaf sample was determined by DPPH assay. The IC_{50} values of watery and ethanol extracts were found to be 127 and 38 μg/mL respectively. Ethanol extract of the leaf sample was observed that higher antioxidant activity than watery extract but weaker activity than BHT (IC_{50} of BHT=11.71 μg/mL). Essential oils from the leaf of the sample were extracted by steam distillation method and analyzed by GC-MS spectroscopic method. According to the results, the components (3-carene, D-limonene, *trans*-carveol, D-carvone, 2-cyclohexen-1-one, 3-methyl-6-(1-methylethylidene), beta-bourbonene and alpha-copaene) were investigated in the extracted essential oils of leaf of *Mentha spicata* L. (Pu Si Nan).

Keywords: *Mentha spicata* L., phytochemical tests, nutritional values, antioxidant activity, antimicrobial activity, essential oils

Introduction

Mint, commonly known as "Pudina" in most Indian languages, belongs to the genus *Mentha* in the family Lamiaceae. There are 25-30 species within the genus *Mentha*, including *spearmint*, *peppermint*, *wild mint*, *corn mint*, *curled mint*, *bergamot*, *American mint*, *Korean mint*, etc. of which spearmint is the most common of all (Kumar *et al*., 2006).

Spearmint (*Mentha spicata* L.) belongs to the family Lamiaceae (Tetika *et al.*, 2013). The plants of this family are a rich source of polyphenols and thus possessing strong antioxidant properties (Robinson, 1983).

Mentha spicata possesses several biological activities and is used in folkloric medicine as a carminative, antispasmodic, diuretic, antibacterial, antifungal and antioxidant agent and for treatment of colds and flu, respiratory tract problems, gastralgia, hemorrhoids, and stomachache (Boukef, 1986 and Leporatti and Gheira, 2009).

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Materials and Methods

Collection and Preparation of Plant Sample

In the present research, the leaf of *Mentha spicata* L. was chosen to be studied. The plant of Pu Si Nan was collected from North Dagon Township, Yangon Region on June, 2018. The collected leaves were washed with water to remove impurities. They were dried at room temperature. The dried sample was made to powder in electric grinder and stored in air-tight container. The dried powdered sample was used to investigate for chemical and biological activities.

Botanical Identification of Collected Sample

Botanical name of collected plant was identified by authorized taxonomist at the Botany Department, Dagon University.

Preliminary Phytochemical Test

Phytochemical investigation of the leaf of *Mentha spicata* L. was carried out according to the standard procedures to investigate the presence or absence of phytochemicals such as alkaloids, α-amino acids, carbohydrates, cyanogenic glycosides, flavonoids, glycosides, organic acids, phenolic compounds, reducing sugars, saponins, starch, steroids, tannins and terpenoids (Harborne, 1984 and Marini-Bettolo *et al*., 1981).

Determination of Nutritional Values

Nutritional values such as moisture content, ash content, fat content, fiber content, protein content, carbohydrate content and energy value of the selected sample were determined by AOAC method (AOAC, 1990 and Liu, 2003).

Evaluation of Antimicrobial Activity

The experiment to evaluate the antimicrobial activity of leaf extracts was carried out at the Pharmaceutical Research Laboratory, Biotechnology Research Department (BRD), Kyaukse District, Mandalay Region, Myanmar.

Sample

EtOH, Pet Ether, CHCl3, EtOAc and H2O extracts of Mentha spicata L. leaf

Tested microorganisms

One Gram-negative bacterium (*Escherichia coli*), three Gram-positive bacteria (*Staphylococcus aureus, Enterococcus faecalis* and *Bacillus cereus*) and one fungal strain (*Candida albicans*) were used as the tested microorganisms for this experiment. Some bacterial strains were kindly supported by Public Health Laboratory (PHL), Mandalay.

Procedure

The agar well diffusion method was used for antimicrobial activity evaluation by modifying the method described. Tested microorganisms were inoculated in Mueller Hinton broth at 37 °C for overnight. On the next day, the overnight broth culture was diluted with normal saline to obtain the OD_{600} at 0.08 to 0.1 with the approximate cell density of $1.5x10^8$ CFU/mL. Mueller Hinton agar plates were prepared and sterilized by autoclaving at 121 °C for 15 min. The broth inoculums were evenly spread out with sterile cotton swabs on the Mueller Hinton agar plates to obtain the uniform inoculums. After the plate was inoculated, 8-mm diameter wells were made on the agar

medium by using a sterile cork borer. The wells then filled with 50 µL of different plant extracts with the concentration of 25 mg per 50 µL. Ethanol (70 %) was used to prepare the extracts and as a solvent control. Tetracycline hydrochloride 30 µg/well was used as the positive control. Then, the plates were placed in an incubator at 37 °C for 16 to 18 hours. After incubation, the plates were examined and zone diameters of complete inhibition were measured and recorded to the closest millimeter (Snoussi *et al.,* 2015).

Screening of Antioxidant Activity

DPPH (1, 1-diphenyl-2-picryl-hydrazyl) radical scavenging assay was chosen to assess the antioxidant activity of selected simple. This assay has been widely used to evaluate the free radical scavenging effectiveness of various flavonoids and polyphenols in food system (Leea *et al*., 2002).

In this experiment, the antioxidant activity was studied on 95 % ethanol extract and watery extract from leaf sample.

Procedure

DPPH radical scavenging activity was determined by UV spectro-photometric method. The control solution was prepared by mixing 1.5 mL of 60 µM DPPH solution and 1.5 mL of 95 % ethanol using shaker. The sample solution was also prepared by mixing thoroughly 1.5 mL of 60 µM DPPH solutions and 1.5 mL of test sample solution. The solutions were allowed to stand at room temperature for 30 minutes. After 30 minutes, the absorbance of these solutions was measured at 517 nm by using (UV-2550) UV-Visible spectrophotometer. Absorbance measurements were done in triplicate for each solution and then mean values so obtained were used to calculate percent inhibition of oxidation and the IC_{50} (50 % inhibitory concentration) values were also calculated by linear regressive excel program (Snoussi *et al.,* 2015).

Extraction of Essential Oils

Procedure

Fresh leaves (500 g) of Pu Si Nan were cut into pieces and mixed with 500 mL of distilled water. The mixture was placed in the 1 liter round-bottomed flask and then connected to a steam generator on one side and a water condenser on the other. The distilled water in the flask was heated and a current of steam was passed into the mixture. The mixture of hot vapours was collected and condensed in order to produce a liquid in which the oil and water form two distinct layers. The upper layer of essential oils was carefully drawn out by a syringe and dried over anhydrous Na_2SO_4 and kept in the freezer. This procedure was done about three times to collect more amounts of essential oils (Basim *et al.,* 2000).

Analysis of Essential Oils by GC-MS Spectroscopy

GC conditions

Source temp	-	200 °C
Inlet line temp	-	270 °C
Injector temp	-	250 °C
Injection volume	-	1.0 µL
Column type	-	Elite5 MS (5% diphenyl 95% dimethyl polysiloxane) 30.0 mL, 0.25 mm, 0.25 µm (thickness)
Oven program	-	Flow 20 °C/ min 80 °C to 210 °C hold 1 min Flow 8.0 °C/ min 210 °C to 250 °C hold 2 min Flow 10 °C/ min 250 °C to 280 °C hold 5 min
Carrier gas	-	Helium (flow rate: 1.0 mL/ min)

MS conditions

Mass Range	-	50 to 550 amu
MS mode	-	Full Scan and Selected Ion Monitoring (SIM)
Delay time	-	3.0 min

Results and Discussion

Preliminary phytochemical investigation was carried out to know the types of phytochemical constituents present in the leaf of *Mentha spicata* L. (Pu Si Nan). According to these results, alkaloids, α-amino acids, carbohydrates, cyanogenic glycosides, flavonoids, glycosides, organic acids, phenolic compounds, reducing sugars, saponins, steroids, tannins and terpenoids were found to be present but starch was absent.

The water content was found to be 15.15 %. The ash content was observed as 25.00 %. The main purpose of protein is to build the body and to require by the body can be used to provide. It was found that the protein content was 23.68 %. The fat content was found to be 7.64 %. Fats are important dietary requirement and provide energy. The fiber was observed as 11.49 %. Fiber reduces the risk of type 2 diabetes. The carbohydrate content was determined by subtraction method. If was found to be 17.04 % and energy value was 232 kcal/100 g of the sample. Carbohydrates are major source of fuel for metabolism, being used both as an energy source and in biosynthesis.

In the present work, antimicrobial activity of PE, EtOH, $CHCl_3$, EtOAc and H_2O extracts obtained from the leaf of (Pu Si Nan) was investigated on five different strains of microorganisms such as *Staphylococcus aureus, Bacillus cereus, Enterococcus faecalis, Escherichia coli* and *Candida albicans* by agar well diffusion method. The measurable diameter, including the well diameter, shows the degree of antimicrobial activity. The well diameter is 8 mm in this experiment. The antimicrobial activity was observed for the extracts of PE, EtOH, $CHCl_3$ and EtOAc against on four strains of microorganisms (except *E. coli*). Watery extract did not show antimicrobial activity on *E. coli* and *C. albicans*. The highest inhibition zone was observed 20.50 ± 0.35 mm on *Bacillus cereus* by EtOAc extract of sample leaf.

The absorbance of different concentrations (6.25, 12.5, 25, 50, 100 and 200 µg/mL) of tested sample was measured as 517 nm by using UV-2550 spectrophotometer. It was found that as the concentrations were increased, the absorbance values were decreased. The larger the % RSA indicates the higher antioxidant activity. In contrast, the lower the IC_{50} indicates the more effective antioxidant activity. The antioxidant activity is expressed as % radical scavenging activity (% RSA) and 50 % inhibition concentration (IC_{50}) value (Cheeseman and Slater, 1993). From the experimental results, IC_{50} values of ethanol and watery extracts of leaf of Pu Si Nan were 38 and 127 µg/mL respectively. According to the results, the ethanol extract was found to be more antioxidant potency than watery extract. Antioxidant potency of ethanol and watery extracts were observed that weaker than compared to the potency of standard BHT (IC_{50}=11.71 µg/mL).

From the analysis of essential oils by Gas Chromatography with Mass Spectrometer, it was observed that the phytoconstituents (3-carene, D-limonene, *trans*-carveol, D-carvone, 2-cyclohexen-1-one, 3-methyl-6-(1-methylelthylidene), beta-bourbonene and alpha-copaene) were investigated in the essential oils of the selected sample leaf.

Table 1 Nutritional Values of the Leaf of *Mentha spicata* L. (Pu Si Nan)

No.	Nutrients	Content %
1	Proteins	23.68
2	Ash	25.00
3	Fibers	11.49
4	Water content	15.15
5	Carbohydrates	17.04
6	Fats	7.64
7	Energy value (kcal/100 g)	232

Table 2 Inhibition Zone Diameters of Various Crude Extracts of *Mentha spicata* L. Leaf Against on Five Microorganisms

Sample	Inhibition Zone Diameter (mm)				
	Staphylococcus aureus	***Bacillus cereus***	***Enterococcus faecalis***	***Escherichia coli***	***Candida albicans***
(1) PetEther Extract	15.50 ± 0.35	15.50 ± 0.35	13.50 ± 1.06	0	15.00 ± 0.00
(2) EtOH Extract	12.00 ± 0.00	17.50 ± 0.35	18.00 ± 0.71	0	13.00 ± 0.71
(3) $CHCl_3$ Extract	13.00 ± 0.71	17.50 ± 0.35	15.50 ± 0.35	0	14.50 ± 0.35
(4) EtOAc Extract	15.50 ± 0.35	20.50 ± 0.35	18.00 ± 0.00	0	15.00 ± 0.00
(5) H_2O Extract	11.00 ± 0.00	14.00 ± 1.41	15.00 ± 0.00	0	0
70% Ethanol	0	0	0	0	0
Tetracycline Hydrochloride	11.67 ± 0.19	23.67 ± 0.51	16.00 ± 0.67	27.67 ± 0.19	24.33 ± 0.19

Values are means ± SEM of duplicate results.
Agar well diameter = 8mm

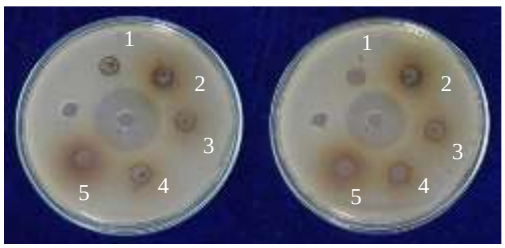

Figure 1 Inhibition zones of various crude extracts against on *Escherichia coli*

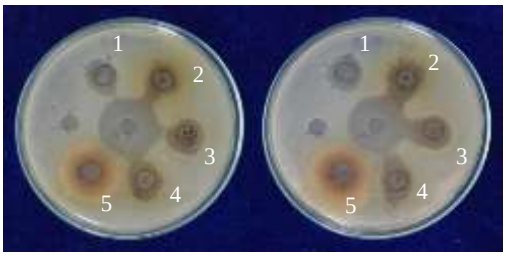

Figure 2 Inhibition zones of various crude extracts against on *Candida albicans*

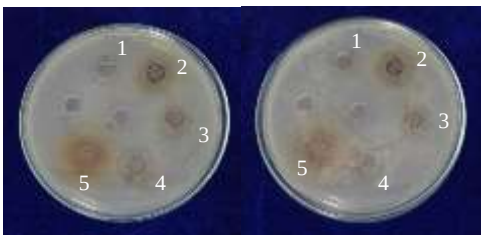

Figure 3 Inhibition zones of various crude extracts against on *Enterococcus faecalis*

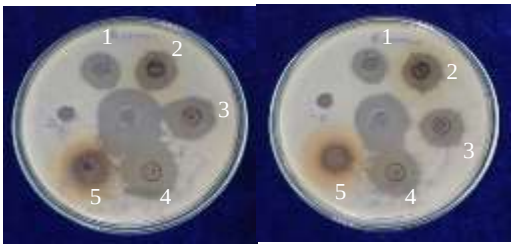

Figure 4 Inhibition zones of various crude extracts against on *Bacillus cereus*

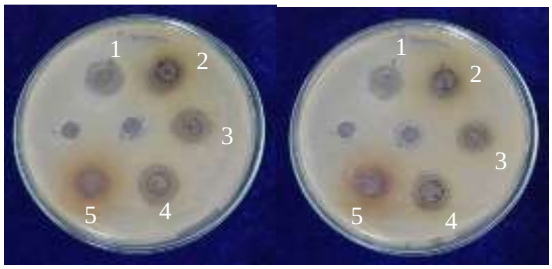

Figure 5 Inhibition zones of various crude extracts against on *Staphylococcus aureus*

Sample Code
(1) = Pet Ether Extract
(2) = EtOH Extract
(3) = $CHCl_3$ Extract
(4) = EtOAc Extract
(5) = H_2O Extract
-C = 70% Ethanol
+C = Tetracycline Hydrochloride

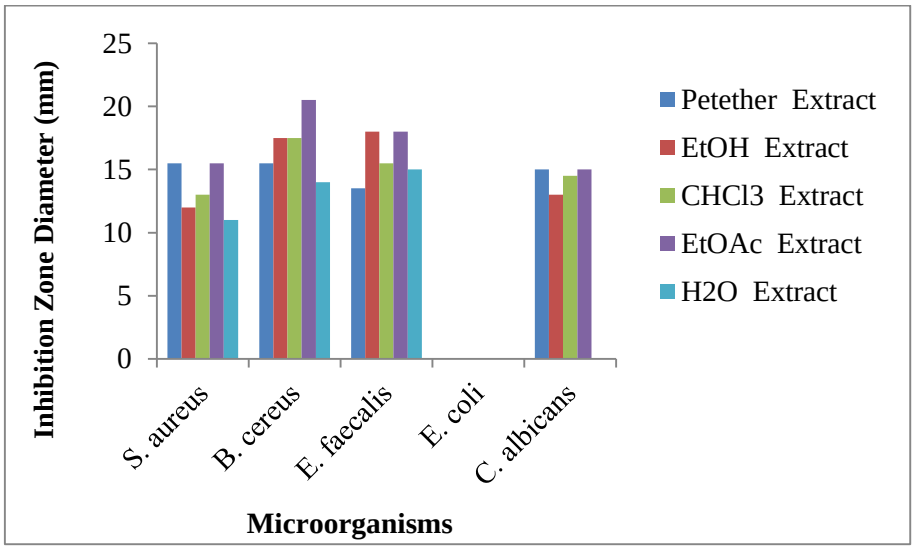

Figure 6 Comparison of inhibition zone diameters for various crude extracts against on five microorganisms

Table 3 Radical Scavenging Activity (% RSA) of Ethanol and Watery Extracts of Leaf of Pu Si Nan and Standard BHT

Extracts	% RSA±SD at Different Concentration (µg/mL)					
	6.25	**12.5**	**25**	**50**	**100**	**200**
EtOH	18.02±003	26.11±0.002	40.02±0.012	58.96±0.005	80.85±0.01	92.68±0.016
H_2O	6.16±0.001	9.1±0.160	16.4±0.002	26.51±0.011	44.98±0.007	63.57±0.003
BHT	33.43±0.010	52.38±0.005	62.14±0.001	68.37±0.011	74.06±0.002	82.34±0.007

Table 4 IC_{50} Values of Ethanol and Watery Extracts of Leaf of Pu Si Nan and Standard BHT

Sample Extracts	IC_{50} (µg/mL)
Ethanol	38
Water	127
BHT	11.71

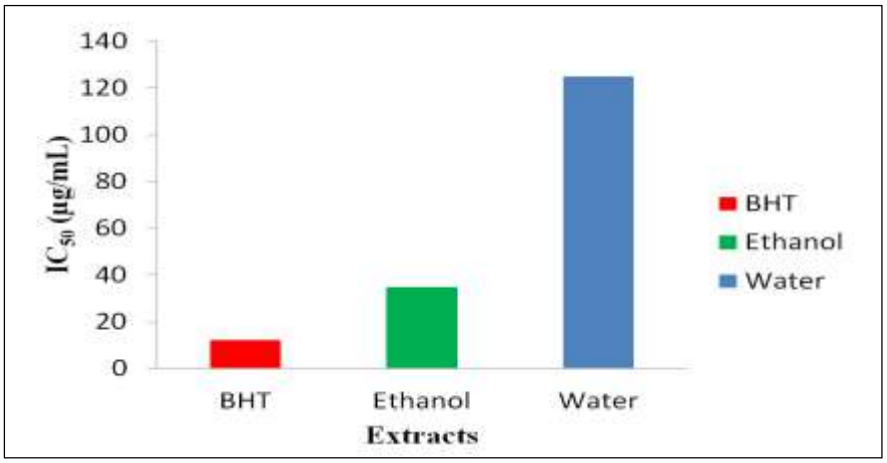

Figure 7 IC_{50} values of ethanol and watery extracts of leaf of Pu Si Nan and standard BHT

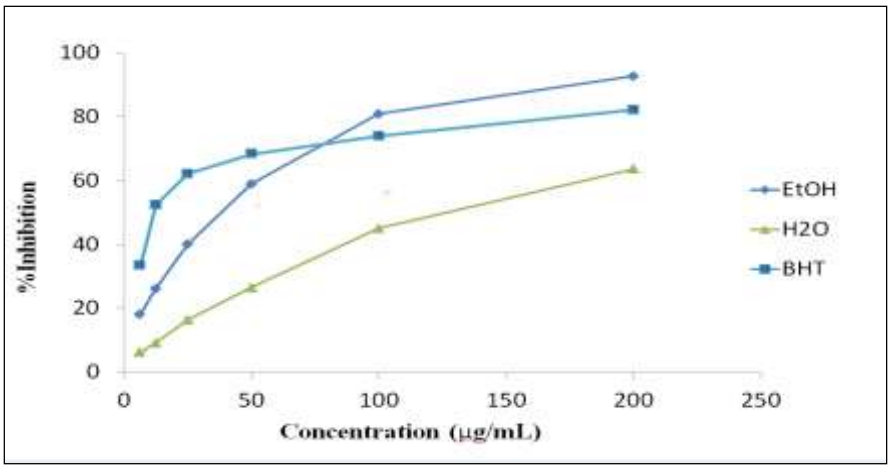

Figure 8 A plot of % RSA of ethanol and watery extracts of leaf of Pu Si Nan and standard BHT on antioxidant activity

Table 5 Phytochemical Constituents in the Essential Oils of Leaf of *Mentha spicata* L. (Pu Si Nan) by GC – MS Spectroscopy

No	
1	3-carene
2	D-limonene
3	*trans*-carveol
4	D-carvone
5	2-cyclohexen-1-one, 3-methyl-6- (1-methylelthylidene)
6	beta-bourbonene
7	alpha-copaene

(a)

(b)

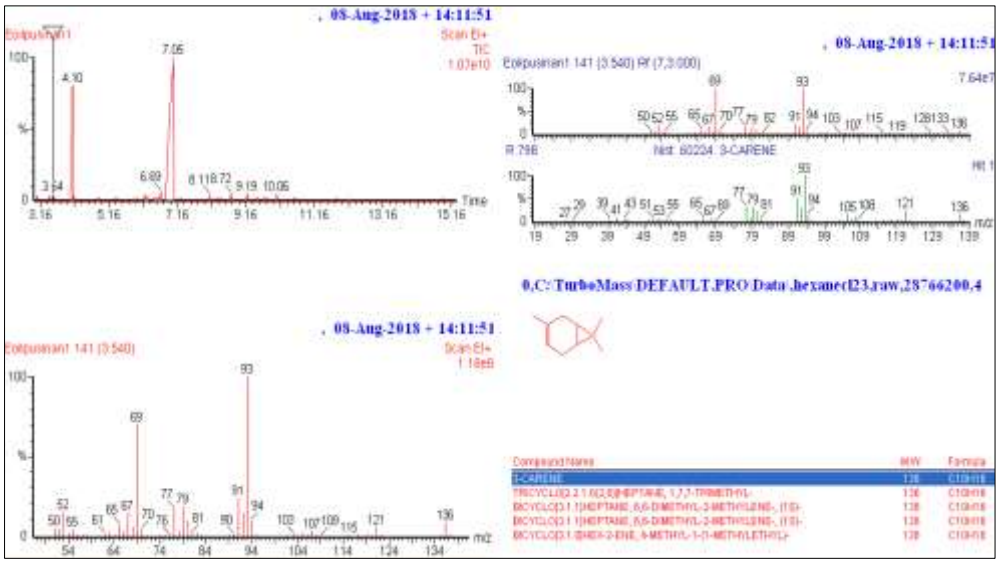

Figure 9 (a) Gas chromatogram and (b) mass spectrum of 3-carene

(a)

(b)

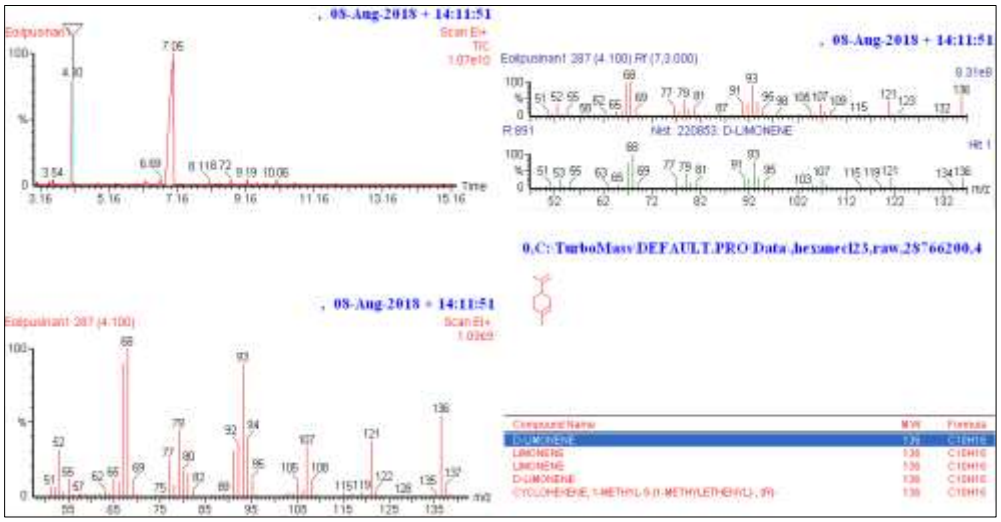

Figure 10 (a) Gas chromatogram and (b) mass spectrum of D-limonene

(a)

(b)

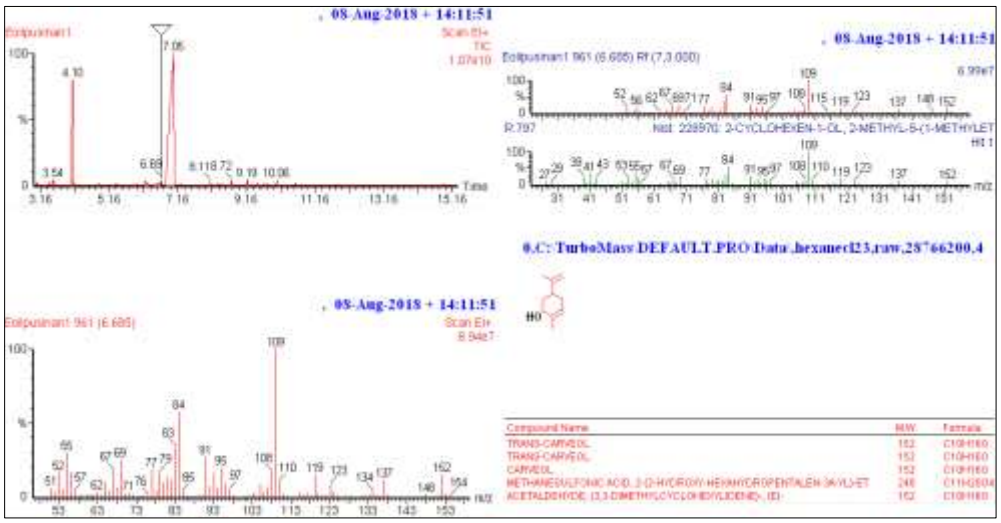

Figure 11 (a) Gas chromatogram and (b) mass spectrum of *trans*-carveol

(a)

(b)

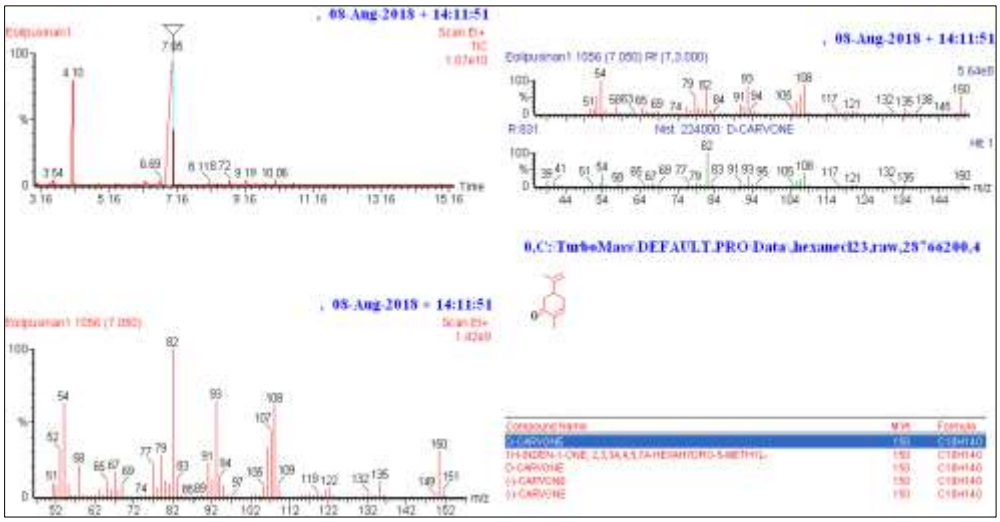

Figure 12 (a) Gas chromatogram and (b) mass spectrum of D-carvone

(a)

(b)

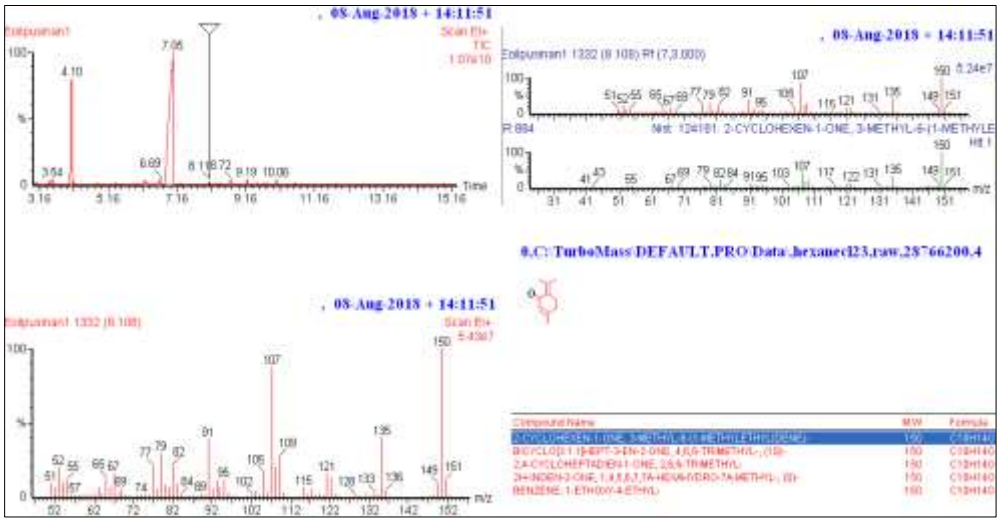

Figure 13 (a) Gas chromatogram and (b) mass spectrum of 2-cyclohexen-1-one, 3-methyl-6 (1-methylethylidene)

(a)

(b)

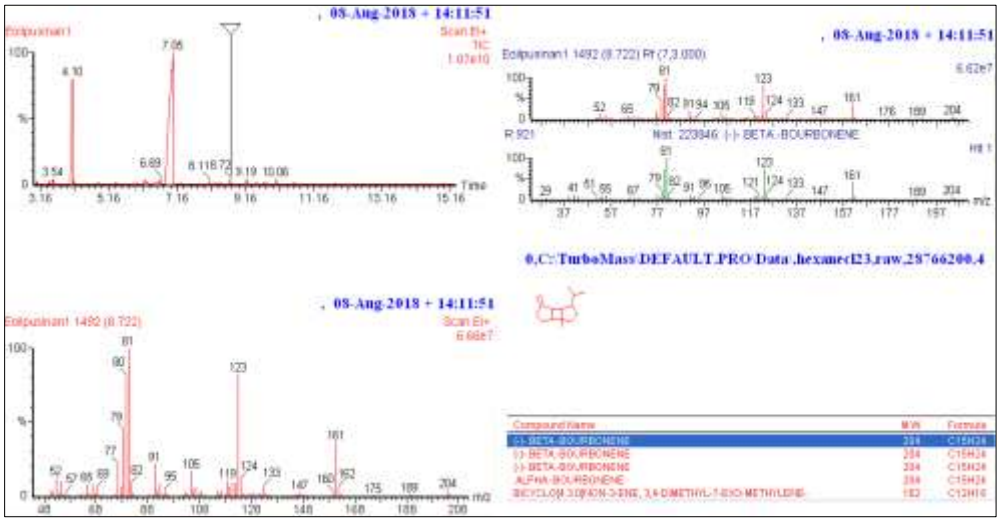

Figure 14 (a) Gas chromatogram and (b) mass spectrum of beta-bourbonene

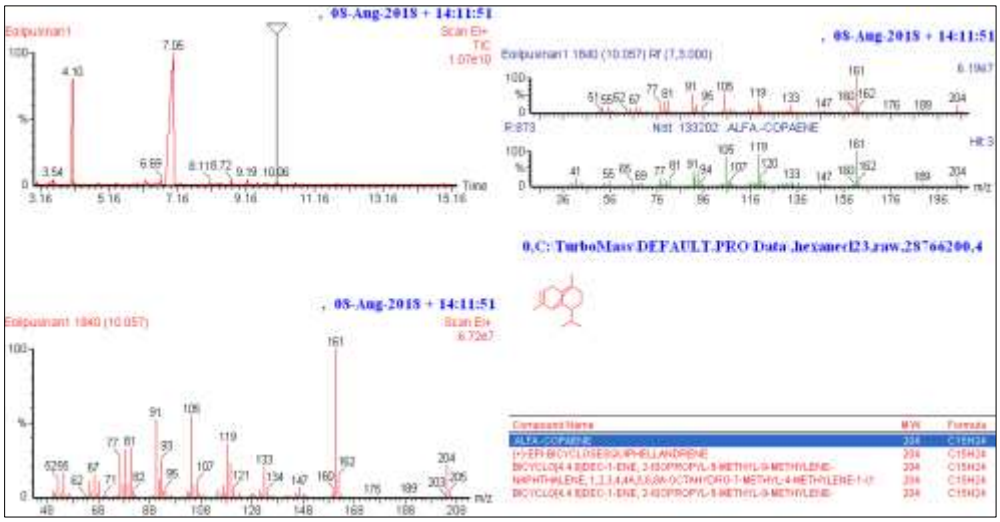

Figure 15 (a) Gas chromatogram and (b) mass spectrum of alpha-copaene

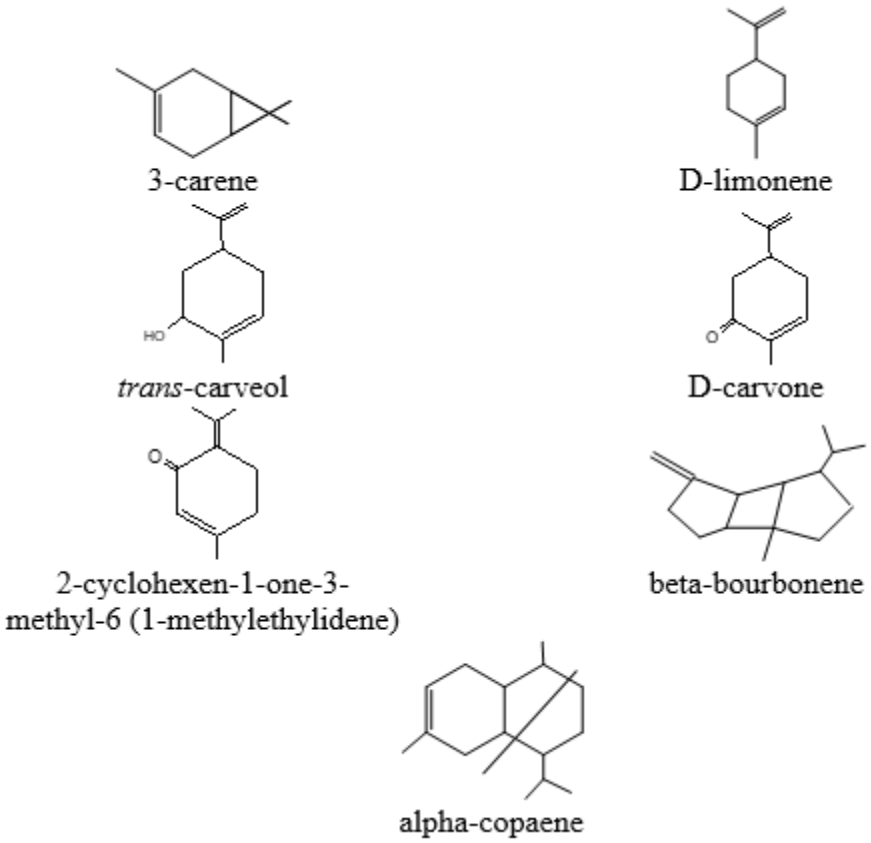

Figure 16 Molecular structures of phytochemical constituents in the essential oils of *Mentha spicata* L. (Pu Si Nan) leaf

Conclusion

From the overall assessment of chemical and biological investigation of the selected plant, *Mentha spicata* L. leaf, the following inferences could be deduced. Preliminary phytochemical tests performed by test tube method indicated that the presence of alkaloids, α-amino acids, carbohydrates, cyanogenic glycosides, flavonoids, glycosides, organic acids, phenolic compounds,

reducing sugars, saponins, steroids, tannins and terpenoids in the leaf of Pu Si Nan whereas starch was absent in the sample.

The nutritional values of the leaf of sample were evaluated by using AOAC method. It was suggested that the sample contained proteins (23.68 %), ash (25.00 %), fibers (11.49 %), water content (15.15 %), carbohydrates (17.04 %), fats (7.64 %) and energy value (232 kcal/100 g). So the leaf of Pu Si Nan is rich source of protein.

The antimicrobial activity of the PE, EtOAc, EtOH, $CHCl_3$ and H_2O extracts of the leaf of Pu Si Nan was screened by using agar well diffusion method against on 5 bacterial strains namely *Staphylococcus aureus, Bacillus cereus, Enterococcus faecalis, Escherichia coli, Candida albicans*. All extracts did not show antimicrobial activity on *E. coli*. Watery extract was not observed activity against on *C. albicans*. PE, EtOH, $CHCl_3$ and EtOAc extracts showed antimicrobial activity on *S. aureus, B. cereus, E. faecalis* and *C. albicans*. EtOAc extract showed highest activity against *Bacillus cereus* (ID = 20.50 ± 0.35mm). The leaf sample can be used to treat diarrhea, emetic toxin, nausea and vomiting.

The antioxidant activity of ethanol and watery extracts of the leaf of Pu Si Nan was evaluated by DPPH free radical scavenging method. In the study of antioxidant activity, the IC_{50} values of ethanol and watery extracts of the leaf part of Pu Si Nan were observed as 38 μg/mL and 127 μg/mL respectively. The ethanol extract of *Mentha spicata* L. leaf was observed that potent antioxidant activity than watery extract but weak activity than standard BHT (IC_{50}= 11.71 μg/mL).

Some phytochemical constituents (3-carene, D-limonene, *trans*-carveol, D-carvone, 2-cyclohexen-1-one, 3-methyl-6-(1-methylethylidene), beta-bourbonene and alpha-copaene were extracted from the essential oils of *Mentha spicata* L. leaf (Pu Si Nan).

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