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GC-MS ANALYSIS AND ANTI-MICROBIAL ACTIVITY OF ESSENTIAL OIL OF *MENTHA PIPERITA* L. FROM KULLU-A NORTH INDIAN REGION OF HIGHER ALTITUDE HIMALAYAS

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ABSTRACT

The essential oil analysis of Mentha piperita L. (Peppermint) has been done for the first time from study area of Northern Indian region of Kullu (1362m) district of Himachal Pradesh. The extraction yield for the essential oil of M. piperita L. was 0.42% for sample M-7. The oil was analyzed by GC-MS, the components of oil were identified by comparing their retention indices and mass spectra fragmentation patterns with those stored on the MS-computer library and also from the published literatures. The major constituents reported from essential oils of M. piperita were: L-Menthone (28.66%); Menthol (9.94%); Piperitone oxide (16.0%); Eucalyptol (7.03%); L-Menthone (3.13%); Isoneomenthol (2.93%); α-Phellandrene (3.21%); δ-3-Carene (3.27%); dl-Limonene (2.53%); α-Pinene (2.02%), etc. Furthermore, anti-microbial activity of oil was evaluated using agar well diffusion method. The anti-microbial test results showed that the oil had a potential anti-microbial activity against all seven Gram+ve and Gram-ve bacterial strains such as: Pseudomonas fluorescence, Salmonella typhimurium, Staphylococcus aureus, Escherichia coli, Bacillus subtilis, Staphylococcus epidermis and Acenetobactor calcoaceticus. Essential oil showed maximum zone of inhibition and minimal inhibition concentration against Bacillus subtilis (MTCC-2451) and Pseudomonas fluorescence (MTCC-664) bacterial strains.

Keywords: *Mentha piperita L., Peppermint, Essential oil, GC-MS, Anti-microbial, Kullu (1362m), North India.*

Introduction

Mentha piperita L. (Peppermint) is a medicinally important plant that belongs to the family Lamiaceae (Kirethekar and Basu, 1985). This herb is frequently growing and cultivated in Europe, Asia, North America, Austraia and also in India for the production of peppermint oil. Peppermint is a non-native herbaceous plant, it is a perennial, which can reach up to 100 cm in height and has four-sided stem. The flowers are irregular in shape; they are pinkish or purplish (Clark and Menory, 1980).

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Mentha piperita L. is a hybrid mint which originated probably due to accidental hybridization between *M. aquatica* and *M. spicata*. It is adapted to almost all areas and can be found in different altitudes. However, different hybridization experiments were carried out between *M. piperita* and other species of mints using techniques such as protoplast fusions (Krasnyanski *et al*., 1998; Sato *et al*., 1996).

The volatile oil extracted from the aerial parts of this herb is a source of commercial menthol. Peppermint leaves contain volatile oil that is composed of free menthol, monoterpene, menthofurane and traces of jasmine, which improve the oil's quality remarkably (Dew and Evans, 1984). The essential oil of this

species is most popular and widely used. It is employed for flavoring, pharmaceuticals, mouth washes, cough drops and confectionery. The oil has also antiseptic and local anesthetic properties. Peppermint oil or peppermint tea is often used to treat indigestion; it may also increase the flow of bile from the gall bladder (Mimica *et al.,* 2003; Forster, 1996). Peppermint oils relaxing action acts as counterirritant and analgesic with the ability to reduce pain and improve blood flow to the affected area. Peppermint oil and menthol have moderate antibacterial effects against both Gram-positive and Gram-negative bacteria (Diaz *et al*., 1988). Peppermint is also found to have anti-viral and fungicidal activity (Chaumont and Senet, 1978). Menthol is virucidal against influenza, herpes and other viruses. Aqueous extracts of peppermint leaves were anti-viral against influenza A, newcastle disease virus in egg and cell culture system was studied by Hirobe *et al*., (1994). Beside these, peppermint oil has been the subject of numerous other studies: Baser *et al*., (1999) studied the essential oils of *Mentha* species from Northern Turkey; Lawrence (1997) also reported the essential oils of peppermint; Gerherman *et al*., (2000) study the comparative analysis of some active principles of herb plants including *M. piperita* by GC-MS. Aflatuni *et al*., (2000) studied the variation in the extract composition of mints of different origin cultivated in Finland. A comparative investigation on the essential oil composition of two Bulgarian cultivars of *M. piperita* L. was also carried out (Stojanova *et al*., 2000. Maffei (1999) studied the sustainable methods for a sustainable production of peppermint essential oil. Monoterpene composition of essential oil from *M. piperita* L. with regard to leaf composition using Solid-Phase Micro-extraction and GC-MS analysis was studied by Rohloff (1999). Variation of chemical composition of essential oil of *M. piperita* L. during the growing time was also investigated by Chalchat *et al*., (1997). Productivity and biochemical composition of *M. piperita* L. of different origins (Lithuanian, Polish and from Ukraine) was also studied by Dambrauskiene *et al*., (2008). Phenols and lactones in Italo-Mitcham peppermint oil *M. piperita* L. were studied by Naf and Velluz (1998).

As a part of our investigation on aromatic medicinal plants, the aim of this work is to provide more information on the composition of essential oil obtained from *M. piperita* L. from a naturally grown species, collected from Kullu (1362m) of Himachal Pradesh region of Northern India. Thus, it is the first record of analysis of essential oil along with antimicrobial activity of *M. piperita* L. from the study area.

Experimental

Plant material

Fresh leaves of *Mentha piperita* L. were collected from Kullu (1362m) of Himachal Pradesh from Northern India, during the month of June, 2008 (Table 1). Specimens were authenticated by the Botanical Survey of India (BSI, Northern Circle), Dehradoon, Department of Biodiversity, I.H.B.T. (CSIR) Palampur and deposited in the herbarium of Department of Botany, Punjabi University, Patiala (Punjab) India.

Oil distillation

Five hundred grams fresh sample of leaves from study area were separated and ground, then immersed in water in a round bottom flask and hydrodistilled for 4h in a full glass Clevenger-type apparatus as recommended by British Pharmacopoeia giving yellowish oils. The essential oil was dried over anhydrous sodium sulphate (Merck) until the last traces of water were removed and then stored in a dark glass bottle at 4 ºC prior to GC-MS analysis (Adams, 1991).

Gas chromatography-Mass-spectrometry

GC-MS (70ev) data were measured on GC-MS (QP 2010 series Shimadzu, Tokyo, Japan) equipped with AOC 20i autosampler and BP-20 capillary column (SGC International Ringwood, Australia) of 30m length, 0.25mm i.d. and 0.25µm film thickness.

Temperature was programmed from 70-220 ºC at a rate of 4 ºC/min, held isothermally at 70 ºC and 220 ºC for 4 and 5 min, respectively. Mass spectrometer source temperature, 200 ºC; interface temperature, 220 ºC; injector temperature, 220 ºC. Sample injection volume 2µL (diluted 5µL oil in 2mL dichloromethane, HPLC grade); split ratio, 1:50 and mass scan, 50-600 amu. Helium was used as a carrier gas with 1.1mL/min flow rate.

Identification of components

The retention index was calculated for all volatile constituents using a homologous series of *n*-alkanes. The components of oil were identified by matching their mass-spectra with those stored in the computer library such as Wiley (McLafferty, 1989), New York mass spectral (MS) library (Jennings and Shibamoto, 1980; Adams, 1989), National Institute of Standards and Technology (NIST) (Stein, 1990) and their retention indices (RI) either with authentic compounds or with published data in the literature based on retention indices of components on same phases of polar columns such as: BP-20, CW-20M, HP-20M and Supelcowax-10.

Microbial strains for anti-microbial activity

The microorganism strains used in the agar disc diffusion method were supplied by the Institute of Microbial Technology, Chandigarh, India. Gram-positive bacteria: *Bacillus subtilis* (MTCC-2451), *Staphylococcus aureus* (MTCC-740), *Staphylococcus epidermis* (MTCC-435), Gram-negative bacteria: *Escherichia coli* (MTCC-443), *Salmonella typhimurium* (MTCC-1251), *Pseudomonas fluorescence* (MTCC-664) and *Acenetobactor calcoaceticus* (MTCC-127).

Anti-microbial screening

In vitro anti-bacterial activity of the *M. piperita* essential oil was studied against seven bacterial strains by the agar well diffusion method as described by Perez *et al*., (1990) with certain modifications. Nutrient agar (Hi Media, India) was used as the bacteriological medium. The anti-bacterial activity of essential oils was taken at different concentrations (10, 20, 30, 40, 50, 60 and 70µL/well). The nutrient agar was melted and cooled to 48-50^{o}C and a standardized inoculum of 1 × 10^{6} CFU/mL, (0.5 McFarland) was then added aseptically to the molten agar and poured into sterile petri dishes to give a solid plate. Wells were prepared in the seeded agar plates. The test compound was introduced in the well (8.5 mm). The plates were incubated overnight at 37^{o}C. The anti-microbial spectrum of the oils was determined for the bacterial species in terms of zone sizes around each well. The diameters of zone of inhibition produced by the agent were compared with those produced by the commercial control antibiotics, 20µL each of amoxicillin and ciprofloxacin (5mg/mL of autoclaved distilled water). These are commonly used anti-biotics to treat infections caused by several Gram-positive and Gram-negative bacteria. For each bacterial strain positive controls were maintained. The experiment was performed three times to minimize the error and the mean values were presented.

Minimal inhibition concentration

The essential oil that exhibited considerable activity was diluted with nutrient broth (1:1) in a series of seven sets of three test tubes for different microorganisms (Aboaba *et al*., 2006). An aliquot of 1mL of the bacterial suspension (1x10^{6}) was inoculated into each tube. The control tubes were inoculated with same quantity of sterile distilled water and 75% ethanol. All tubes were incubated at 37^{o}C for 24hrs. The lowest concentration that did not permit any visible growth when compared with the control was considered as the minimum inhibitory concentration. The contents of all tubes that showed no visible growth were cultured on nutrient agar, incubated at 37^{o}C for 24hrs. The minimum bactericidal concentration was considered as the lowest concentration that could not produce a single bacterial colony.

Results and Discussion

The extraction yield for the essential oils of *M. piperita* L. from Kullu (1362m) was 0.42% (M-7) for the sample. The essential oil analysis led to the identification of 28 constituents representing 83.06% of the compositions of oil. The GC-MS chromatograph showing different peaks of the essential oil constituents (Figure 1).

In the essential oil sample, some major constituents reported were as follows: L-Menthone (28.66%); Menthol (9.94%); Piperitone oxide (16.0%); Eucalyptol (7.03%); L-Menthone (3.13%); Isoneomenthol (2.93%); α-Phellandrene (3.21%); δ-3-Carene (3.27%); dl-Limonene (2.53%); α-Pinene (2.02%) and some unidentified compounds with high percentage such as: 5-Methyl-2-(1-methylethylidene)-cyclohexanone (5.98%) was also reported along with some minor constituents (Table 2).

Previous investigations on *M. piperita* oil composition are consistent with our results in which menthone and menthol was found to be the major compounds (Lawrence 1997; Gerherman *et al*., 2000; Aflatuni *et al*., 2000; Stojanova *et al*., 2000; Maffei, 1999; Rohloff, 1999; Chalchat *et al*., 1997; Spencer *et al*., 1997).

Anti-microbial activity showed that, the inhibition zones were found increased considerably when the concentration rate increased. Therefore it can be said that quantity of the oil was important for inhibition effect. Among all Gram-positive bacterial growths, the maximum zone of inhibition was recorded against *Bacillus subtilis* (MTCC-2451) i.e. 61.9mm, followed by 37.6mm in *Staphylococcus aureus* (MTCC-740) and 32.8mm in *Staphylococcus epidermis* (MTCC-435) at 70µL/well (Figures 2, 3).

On the other hand four different Gram-negative bacterial strains were tested and among these microorganisms, *Pseudomonas fluorescence* (MTCC-664) showed maximum zone of inhibition i.e. 54.7mm, followed by *Acenetobactor calcoaceticus* (MTCC-127) i.e. 37.1mm. The minimum zone of inhibition was recorded against the *Salmonella typhimurium* (MTCC-1251) strain i.e. 29.9mm (Table 3).

The minimal inhibition concentration (MIC) was 2µL recorded in Gram-negative strain *Pseudomonas fluorescence* (MTCC-664) (Figure 4) followed by a Gram-positive strains *Bacillus subtilis* (MTCC-2451) 2.5µL (Table 4).

From these, it is concluded that the essential oil showed maximum zone of inhibition and minimal inhibition concentration against *Bacillus subtilis* (MTCC-2451) and *Pseudomonas fluorescence* (MTCC-664) bacterial strains, which indicate that *M. piperita* L. essential oil has capacity to inhibit the growth of both Gram-positive and Gram-negative bacterial strains when used in a higher amount. Further, on the basis of previous studies on *Mentha* genus and present results of *M. piperita* L., which proved that, it is a medicinal and aromatic plant that acts as an important anti-microbial agent against many gram-positive and gram-negative bacterial strains and also has higher percentage of some most important chemical constituents.

According to Fleming (1998) reported that, due to many potent compounds such as menthol, menthone, limonene, etc., in *M. piperita* shows significant anti-microbial activity. These compounds have higher medicinal value especially in the treatment of dyspepsia, epigastric bloating, impaired digestion. Deans and Baratta (1998) also investigated that the compounds from *M. piperita* possess anti-microbial activity and suggesting that the *M. piperita* leaf extracts should contains the effective active constituents responsible for eliminating the bacterial pathogens.

Therefore, finally, it can be concluded that the active chemical compounds present in *M. piperita* L. should find place in treatment of various bacterial infections. The results from the present investigation are very encouraging and indicate that herb should be studied more extensively to explore its potential in the treatment of infectious diseases as well.

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Table 1. Collection details and essential oil yield of *M. piperita* L. from study area of North Indian, Himachal Pradesh, Himalayas.

Species name	Place of collection	Herbarium Number	Altitude of study area (m)	Month & year of collection	Oil yield (%)
Mentha piperita L.	Kullu (H.P.) North India	52131 (PUN)*	1362	June, 2008	0.42

*PUN: Abbreviation for Herbarium, Department of Botany, Punjabi University, Patiala, as indicated in Index Herbariorum.

Table 2. Volatile oil composition of *M. piperita* L. (M-7) from Kullu (1362 m).

S. No.	RT[a]	Constituents	RI[b]	RI[c]	RA[d]
1.	3.666	α-Terpinene	---	1287	0.59
2.	3.958	dl-Limonene	---	1154	2.53
3.	4.140	Eucalyptol	---	1206	7.03
4.	4.571	α-Pinene	---	1039	2.02
5.	4.776	δ-3-Carene	1200	1147	3.27
6.	4.909	δ-3-Carene	1206	1147	0.11
7.	5.034	3-Octanone	1211	1205	0.12
8.	5.307	α-Phellandrene	1222	1216	3.21
9.	5.556	2-Methylbutyl-2-methylbutyrate	1232	---	0.12
10.	6.944	Octyl cyclobutanecarboxylate	1290	---	0.30
11.	7.203	Artemisia ketone	1300	1333	0.05
12.	8.564	3-Octanol	1347	1382	1.08
13.	10.405	L-Menthone	1408	1456	28.66
14.	10.584	*trans*-Sabinene hydrate	1414	1465	0.39
15.	11.156	L-Menthone	1432	1456	3.13
16.	13.165	L-Linalool	1495	1538	0.50
17.	13.351	neo-Menthol acetate	1501	---	0.38
18.	13.561	*cis*-Isopulegone	1508	1580	0.26
19.	13.650	Isopulegol	1511	1573	0.09
20.	13.975	Isopulegone	1521	1533	0.05
21.	14.068	*trans*-Caryophyllene	1523	---	0.91
22.	14.450	Isoneomenthol	1537	1622	2.93
23.	14.579	*trans*-Sabinene hydrate	1541	1465	0.19
24.	15.338	neo-Menthol	1566	1599	0.07
25.	15.727	5-Methyl-2-(1-methylethylidene)-cyclohexanone	1579	---	5.98
26.	15.780	Menthol	1580	1612	9.94
27.	16.693	δ-Terpineol	1610	1655	0.10
28.	17.398	Borneol	1634	1653	1.63
29.	17.629	Piperitone oxide	1641	1700	4.90
30.	18.111	Piperitone	1657	1697	0.34
31.	18.216	Piperitone oxide	1661	1700	9.19
32.	18.459	p-Menthane-1,2,3-triol	1669	---	0.33
33.	19.399	Perilla acetate	1700	---	0.15
34.	24.389	Piperitone oxide	1797	1700	0.17
35.	30.288	Thymol	2102	2115	0.51
36.	37.012	5-Hydroxy-7-methoxy-2-methyl-chromone	2290	---	5.24
37.	40.624	Methyl linolenate	2557	---	0.13
38.	41.808	3,3-Isopropyledenebis(1,5,8,11-tetraoxacyclotridecane)	2614	---	0.07
39.	43.507	Octadecanoic acid	---	---	0.77
40.	44.773	Angecin	---	---	2.58

RT[a] : Retention time.
RI[b] : Retention indices according to their elution order on BP-20 polar column.

RI[c] : Actual retention indices of components on same phases of columns (BP-20, CW-20M, HP-20M and Supelcowax-10).
RA[d] : Percentage of components.
--- : RI cannot be calculated.

Table 3: Anti-microbial activity of essential oil of *M. piperita* L. against Gram-positive and Gram-negative bacterial strains.

S. No.	Nature of Bacterial Strains	Microorganisms (Bacterial Source Number)	Diameter of inhibition zone (mm) of essential oil concentration used for anti-microbial analysis (µL/well) (*n=3*)							Control +ve (*n=3*)	
										Amoxicillin 5mg/mL	Ciprofloxacin 5mg/ mL
			10µL	20µL	30µL	40µL	50µL	60µL	70µL	20µL	20µL
1.	Gram +ve	*Bacillus subtilis* (MTCC-2451)	6.1	11.3	20.2	35.7	47.2	56.3	61.9	42	51
2.		*Staphylococcus aureus* (MTCC-740)	2.4	3.9	8.0	19.2	27.7	33.5	37.6	34	43
3.		*Staphylococcus epidermis* (MTCC-435)	2.9	5.3	7.9	16.5	23.3	27.1	32.8	32	56
4.	Gram -ve	*Escherichia coli* (MTCC-443)	1.8	2.9	7.2	12.8	21.1	29.0	32.2	46	52
5.		*Salmonella typhimurium* (MTCC-1251)	N.A	3.7	10.1	14.4	19.2	28.1	29.9	26	39
6.		*Pseudomonas fluorescence* (MTCC-664)	4.6	7.2	17.4	26.7	38.4	46.7	54.7	41	58
7.		*Acenetobactor calcoaceticus* (MTCC- 127)	N.A	3.1	9.7	19.2	26.7	31.4	37.1	39	46

All values are mean of triplicates (*n=3*); Gram +ve: Gram-positive; Gram –ve: Gram-negative; N. A: No Activity.

Table 4. Minimal inhibition concentration (MIC) (µL) of essential oil of *M. piperita* L.

S. No.	Nature of Bacteria	Microorganisms (Bacterial Source Number)	MIC (µL)
1.	Gram +ve	*Bacillus subtilis* (MTCC-2451)	2.5
2.		*Staphylococcus aureus* (MTCC-740)	5
3.		*Staphylococcus epidermis* (MTCC-435)	4.5
4.	Gram-ve	*Escherichia coli* (MTCC-443)	6
5.		*Salmonella typhimurium* (MTCC-1251)	14
6.		*Pseudomonas fluorescence* (MTCC-664)	2
7.		*Acenetobactor calcoaceticus* (MTCC-127)	15

MIC: Minimal Inhibition Concentration; Gram +ve: Gram-positive; Gram –ve: Gram-negative.

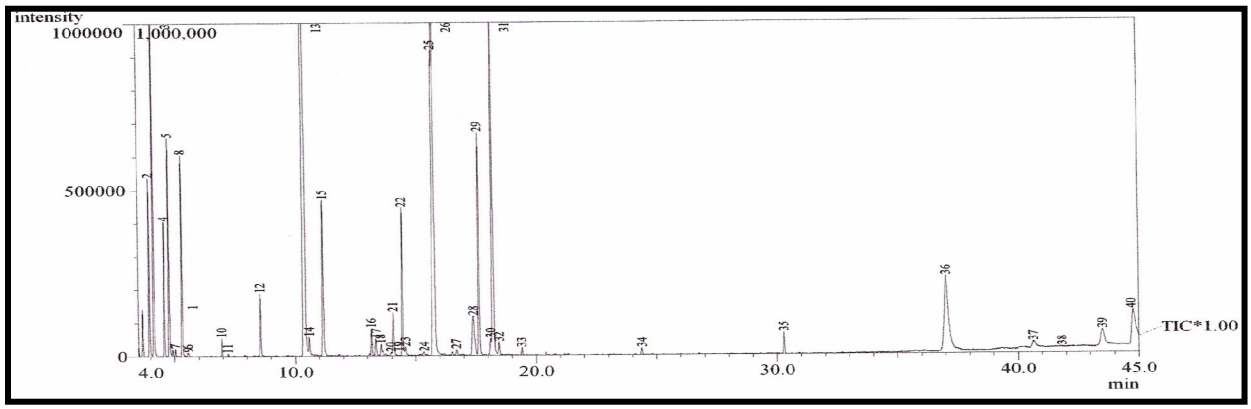

Figure 1. GC-MS chromatogram of *M. piperita* L. (M-7) collected from Kullu (1362m).

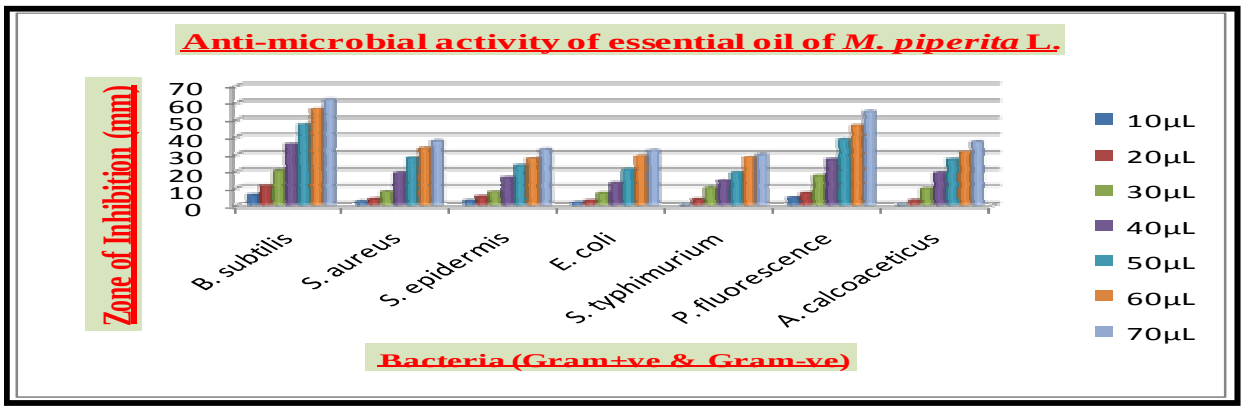

Figure 2. Bar diagram showing anti-microbial activity of essential oil against bacteria.

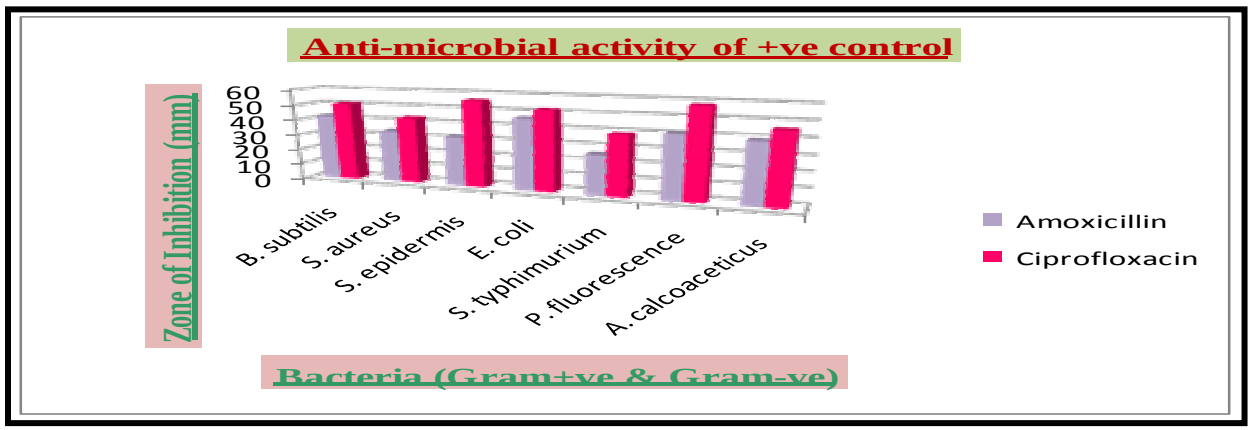

Figure 3. Bar diagram showing anti-microbial activity of +ve controls against bacteria.

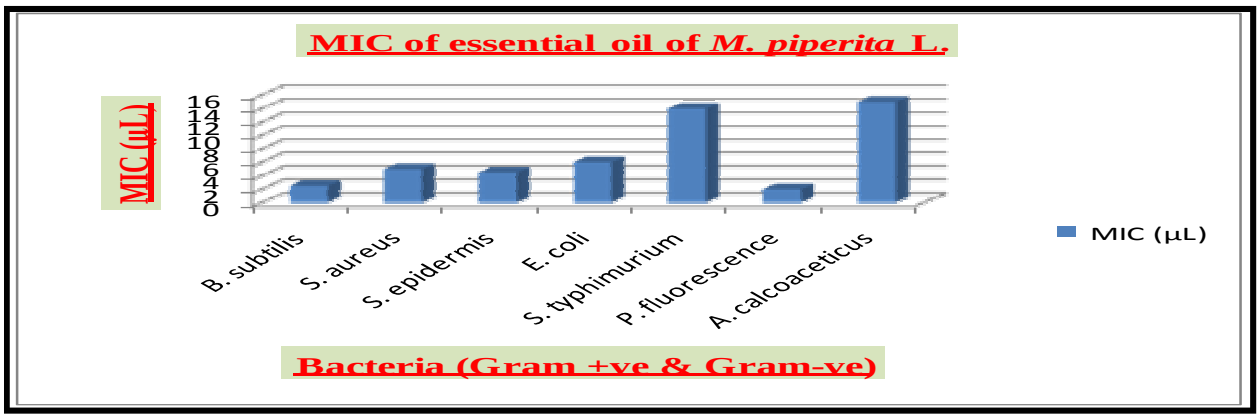

Figure 4. Bar diagram showing Minimum Inhibitory Concentration against bacteria.

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