REPORT

Antimicrobial potentials of *Mentha longifolia* by disc diffusion method

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Abstract: This study was conducted for the assessment of the antimicrobial activities of different solvents extracted samples from the aerial parts of *Mentha longifolia* against ten microbial species through the disc diffusion assay using two different concentrations of 1 and 2 mg disc[1]. All extracts from *Mentha longifolia* showed different ranges of antimicrobial activities. Butanol and ethyl acetate fractions showed inhibitory activities against all microbial species. Methanol fraction showed inhibitory effects against all the tested microbial species except *Salmonella typhi*. *Salmonella typhi* was also not controlled by methanol, petroleum ether and dichloromethane extracted samples. The most susceptible gram positive bacteria was *Bacillus atropheus* and *Bacillus subtilis* and were inhibited by all extracts and *Staphylococus aureus* was least susceptible among gram positive bacteria. *Klebsiella pneumoniae* was the most susceptible gram negative bacterium and *Salmonella typhi* was highly resistant among the gram negative bacteria. *Erwinia carotovora* and *Agrobacterium tumefaciene* were susceptible to all fractions. All fractions showed antifungal activities against *Candida albicans* except water extracted samples.

Keywords: Antimicrobial, *Mentha longifolia*, disc diffusion, antifungal.

INTRODUCTION

Different contiguous diseases and drug-resistant microorganisms is a serious challenge to human health through out the world. Resistance to antibiotics by various microbes has posed difficulties in the development and discoveries of new drugs for their control (Russell, 2002). As results, herbal medicines containing novel antimicrobial therapeutic agents are gaining importance (Goots, 1990; Adekunle and adekunle, 2009; Shad *et al*., 2013). Herbal medicines are simple, more specific and bio-degradable with very fewer side effects (Chin *et al*., 2006). Phytochemicals provides an opportunity for structural diversity and biological functionality which is considered very important for drug discovery and development (Nisbet, 1997; Verpoorte, 2000). Medicinal plants are a good bio-source of traditional drugs, modern medicines, nutraceuticles, food supplements and chemical basis for synthetic drugs (Hammer *et al.*, 1999; Taylor *et al.*, 2001; Kubmarawa *et al.*, 2007; Zahin *et al.*, 2010; Bakht *et al*., 2011 a,b,c,d; 2012 a,,b; 2013 a,b).

Mentha longifolia L. (common name; wild mint or horse mint) belong family Lamiacea. In traditional medicine, the leaves of mint are used for the treatment of minor sore throat, aches and in nasal decongestants. In addition, horsemint possesses antiseptic properties and its beneficial effect on the digestion (Al-Bayati, 2009). Tea prepared from the leaves is traditionally used for the treatment of fevers, headaches and digestive discomfort. Essential oils of mints are known to act as antimicrobial, antispasmodic, carminative and antiviral agents (Daferera *et al.,* 2003). Phenolic compounds found in mints are found to possess a wide range of pharmacological activity. In addition, Mentha *spp*. has been used as a traditional remedy for nausea, bronchitis, flatulence, anorexia, ulcerative colitis, and liver complaints (Cowan, 1999; Iscan *et al.,* 2002; Moreno *et al.,* 2002). The present study was conducted to investigate the antimicrobial activity of different solvents extracted samples from *Mentha longifolia* against different micro-organisms.

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MATERIALS AND METHODS

Plant materials

Aerial parts of *Mentha longifolia* were collected during the month of April from different localities of Mardan district of Khyber Pukhtunkhwa province Pakistan. The collected plants were thoroughly washed with tape water to remove the dirt and soil particles. The clean aerial parts of *Mentha longifolia* were dried in a shaded room for a period of 7 days.

Crude extract preparation

Shade dried aerial parts of *Mentha longifolia* were chopped and grinded and 450 grams of dried powder were kept in methanol at room temperature for 6 days. The methanol soluble compounds were filtered (Whatman ^{TM}) three times and subjected to rotary evaporator (Rotavapor ^{R}-R 210/R215; BUCHIL Labortechnik AG) for drying. The semisolid extract was dried at 45ºC in water bath

yielding about 65 grams of dried crude (methanol) extract.

Fractionation of crude extract

Crude extract prepared was divided into two portions, one portion (10 g) was poured into a glass vials to be tested as crude methanol extract for antimicrobial activity. The second portion (55 g) was dissolved in water and distilled petroleum ether was added into it. Compounds soluble petroleum ether phase were collected and the lower aqueous phase was extracted thrice with petroleum ether. All fractions of petroleum ether were combined and dried via rotary evaporator leaving behind semisolid petroleum ether fraction. The semisolid petroleum ether fraction was dried at 45ºC. The same process of fractionation was carried out for dichloromethane, ethyl acetate and butanol respectively resulting in dichloromethane, ethyl acetate and butanol fractions. The lower aqueous phase at the end of the process was taken and dried as described earlier.

Culture media and its preparation

Nutrient agar media (HiMedia Laboratories Pvt. Ltd.) was used for the culturing and growth and nutrient broth for shaking incubation and standardization of different microorganisms ((Bakht *et al.,* 2011 a, b, c, d and 2012). Media was prepared as described by Bakht *et al.* (2011 a, b, c, d and 2012).

Microorganisms used

Antimicrobial activity of different solvent extracted samples of *Mentha longifolia* was tested against the following different bacterial and fungal strains (table 1).

Disc diffusion susceptibility method

Disc diffusion assay was carried out as described in Bakht *et al.* (2011 a, b, c and 2012). Briefly, nutrient agar media plates were seeded with 18-24 hrs cultures of microbial inoculums (a standardized inoculums 1-2 $\times 10^7$CFU ml^{-1} 0.5 McFarland Standard). Wattman No.1 filter paper discs (6mm in diameter) were placed with the help of a sterile forceps on the media and then plant extracts in concentrations of 1 and 2 mg $disc^{-1}$ in 6 and 12 µl volume were applied on the discs. Antibiotics (6 µl $disc^{-1}$) as positive control and DMSO (6 µl $disc^{-1}$) as negative control were also applied on the discs. Inoculated plates were then incubated at 37 ºC for 18-24 hrs. The next day zones of inhibition were recorded in mm around the discs in each plate.

Positive controls

For Gram positive bacteria;	Azithromycin 50 µg 6 $µl^{-1}$
For Gram negative bacteria;	Ciprofloxacin 30 µg 6 $µl^{-1}$
For *Candida albicans*;	Clotrimazole 50 µg 6 $µl^{-1}$

RESULTS

Fig. 1 shows the antibacterial activities of different solvent extracted samples of *Mentha longifolia* against *Bacillus subtilis* by disc diffusion susceptibility method. *Bacillus subtilis* was susceptible to all extracts from *Mentha longifolia* and showed different ranges of antibacterial activities against this bacterium. Ethyl acetate fraction was found to be the most effective and showed 50% inhibitory effects at 2 mg $disc^{-1}$ concentration followed by dichloromethane (39% Zone of Inhibition (ZI) at the same concentration. Water extracted samples were least effective against *B subtilis* and reduced bacterial growth by 21% at 1mg $disc^{-1}$ and 25% at 2 mg $disc^{-1}$. Petroleum ether and butanol extracted samples were almost equally effective against *B subtilis* and reduced the growth by 34% at higher concentration (2 mg $disc^{-1}$). These results agree with Sokmen *et al.* (2000). Petroleum ether, butanol, dichloromethane and methanol extracted samples had almost the same inhibitory activities against *S aureus* (fig. 2). All extracts were moderately effective in the inhibition of *S aureus* at both concentrations i.e. 1 and 2 mg $disc^{-1}$ and reduced the growth of *S aureus* by 30% at 2 mg $disc^{-1}$ concentration while dichloromethane extracted samples showed 31% inhibitory activity against *S aureus* at 2 mg $disc^{-1}$ concentration. On the other hand, water extracts was ineffective in the inhibition of *S aureus* at 1 mg $disc^{-1}$, however, at higher concentration (2 mg $disc^{-1}$) 23% inhibition was noted. Ethyl acetate extracted samples were more effective and reduced the growth of *S aureus* by 36% and 43% at 1 and 2 mg $disc^{-1}$ respectively.

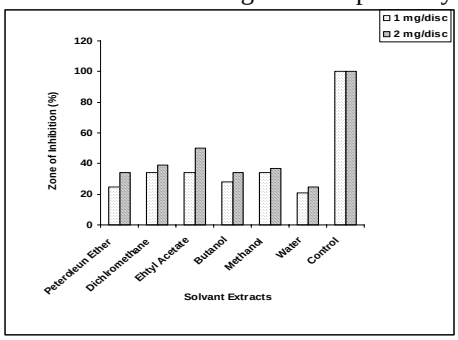

Fig. 1: Antibacterial activity of petroleum ether, ethyl acetate, chloroform, butanol, ethanol and water extracted samples from *Mentha longifolia* against *Bacillus subtilis* by disc diffusion assay.

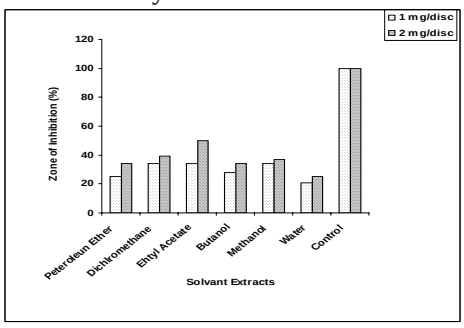

Fig. 2: Antibacterial activity of petroleum ether, ethyl acetate, chloroform, butanol, ethanol and water extracted samples from *Mentha longifolia* against *S aureus* by disc diffusion assay.

Table 1: Microbial strains used in the present study

Microbial species	Gram Strain type	Details of microbial strains used
Bacillus subtilis	Positive	Clinical isolate obtained from Microbiology Laboratory of Quaid-e-Azam University, Islamabad
Candida albicans	Fungus	Clinical isolate obtained from Hayatabad Medical Complex, Peshawar, KPK
Erwinia carotovora	Negative	Plant Pathology department of KPK Agricultural University, Peshawar
Escherichia coli	Negative	ATCC# 25922
Klebsiella pneumoniae	Negative	Clinical isolate obtained from Microbiology Laboratory Quaid-e-Azam University, Islamabad
Pseudomonas aeruginosa	Negative	ATCC# 9721
Salmonella typhi	Negative	Clinical isolate obtained from Microbiology Laboratory, Quaid-e-Azam University, Islamabad
Staphylococcus aureus	Positive	ATCC# 6538
Bacillus atropheus	Positive	
Agrobacterium tumefacien	Negative	

Petroleum ether extracts of the plant was ineffective against *E coli* when applied at lower concentration i.e. 1 mg disc^{-1}, however, when concentration of the sample was increased to 2 mg disc^{-1} inhibitory activity of 20% was observed (fig. 3). *E coli* were found to be highly resistant to dichloromethane fractions and did not show antibacterial activity at both concentrations. Ethyl acetate extracted samples were highly effective among all extracts and reduced the growth of *E coli* by 32% and 44% at lower and high concentration respectively. The antibacterial activity of butanol fraction was measured to be 29% at 2 mg disc^{-1} and that of methanol was 26% at the same concentration. At lower concentration (1mg disc^{-1}) both fractions showed same inhibitory activity of 23%. Water extracted samples reduced the growth of *E coli* by 23% at higher concentration (fig. 3).

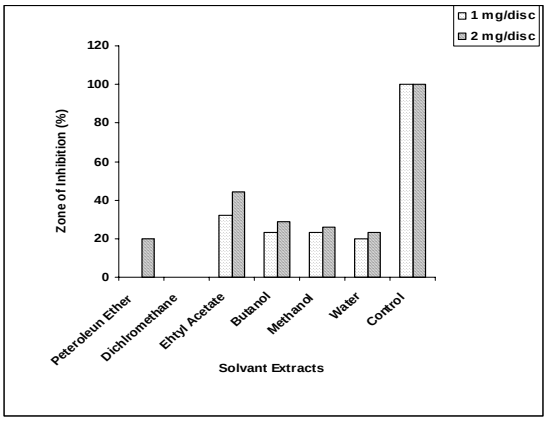

Fig. 3: Antibacterial activity of petroleum ether, ethyl acetate, chloroform, butanol, ethanol and water extracted samples from *Mentha longifolia* against *Escherichia coli* by disc diffusion assay.

S typhi was highly resistant to petroleum ether, dichloromethane and methanol extracted samples and did not show antibacterial activity against at both concentrations (0% ZI). Ethyl acetate fraction reduced the growth of *S typhi* by 37% and 44% at 1 and 2 mg disc^{-1} respectively. Butanol fraction also showed an inhibitory effect of 25% and 33% at 1 and 2 mg disc^{-1} respectively. The inhibitory activity of water extracted samples was found to be 25% and 29% at 1 and 2 mg disc^{-1} respectively (fig. 4).

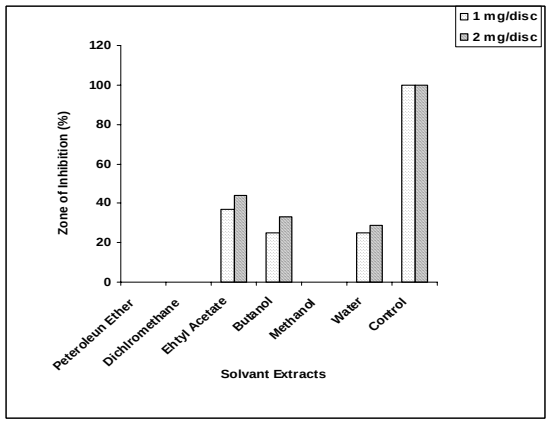

Fig. 4: Antibacterial activity of petroleum ether, ethyl acetate, chloroform, butanol, ethanol and water extracted samples from *Mentha longifolia* against *Salmonella typhi* by disc diffusion assay.

The antifungal activity of different solvents extracted samples from *Mentha longifolia* against the *Candida albicans* in fig. 5. Ethyl acetate fraction showed 55% inhibition in the growth of *Candida albicans* when applied at higher concentration (2 mg disc^{-1}) and 34% at concentration of 1 mg disc^{-1}. *Candida albicans* showed resistance to low concentration of water extracted

samples, however, when the concentration of sample was increased to 2 mg disc^{-1}, 24% reduction in its growth was observed. The inhibitory effect of petroleum ether and butanol fraction was similar against the *Candida albicans* and in both cases zone of inhibition was measured up to 24% at 1 mg disc^{-1} and 34% at 2 mg disc^{-1}. Dichloromethane and methanol fractions were also effective against this fungus and both showed an inhibitory activity of 37% at high concentrations (fig. 5).

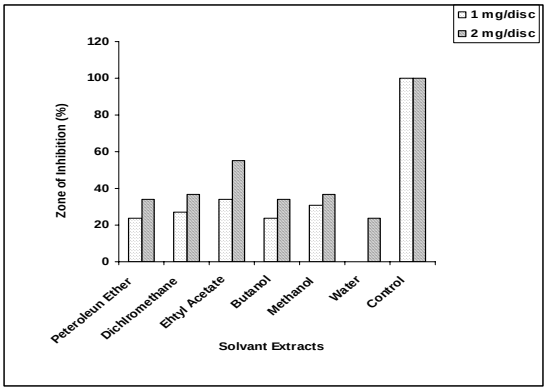

Fig. 5: Antibacterial activity of petroleum ether, ethyl acetate, chloroform, butanol, ethanol and water extracted samples from *Mentha longifolia* against *Candida albicans* by disc diffusion assay.

Fig. 6 shows the antibacterial activity different extracts from *Mentha longifolia* against the growth of *Bacillus atropheus*. Highest activity was observed in case of petroleum ether extracted samples which inhibited the growth by 36% and 54% at 1 and 2 mg disc^{-1} concentration. Dichloromethane fraction was also found to be effective in controlling the growth of *B atropheus* and reduced its growth by 39% at 1 mg disc^{-1} and 45% at 2 mg disc^{-1} concentration. Ethyl acetate also inhibited bacterial growth by 30% and 42% at 1 and 2 mg disc^{-1}. Butanol and water extracted samples showed similar inhibitory activity of 36% at high concentration (2 mg disc^{-1}).

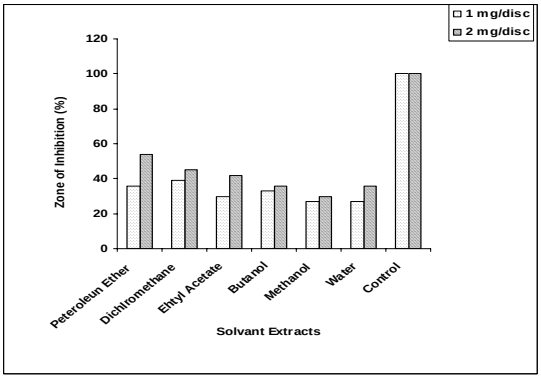

Fig. 6: Antibacterial activity of petroleum ether, ethyl acetate, chloroform, butanol, ethanol and water extracted samples from *Mentha longifolia* against *Bacillus atropheus* by disc diffusion assay.

However, methanol fraction showed antibacterial of 27% at 1 mg disc^{-1} and 30% at 2 mg disc when compared to other extracts. Analysis of the data revealed that *E carotovora* was susceptible to all extracted samples (fig. 7). Highest antibacterial activity was recorded for petroleum ether fraction (37% ZI) followed by dichloromethane, ethyl acetate and methanol samples (34% ZI) at high concentration i.e. 2 mg disc^{-1}. Butanol fraction also exhibited inhibitory activity against *E carotovora* and reduced its growth by 31% at 2 mg disc^{-1}. Water extracts were found to be less effective against *E carotovora* when compared with other samples activity.

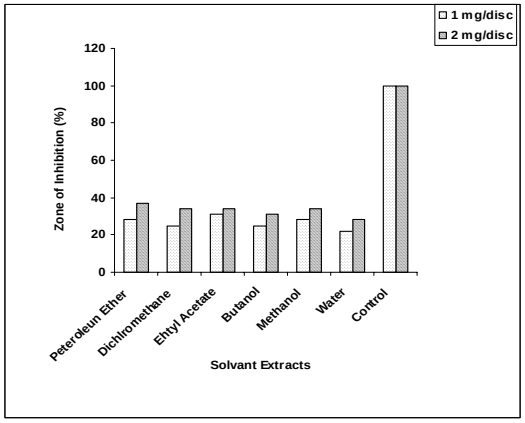

Fig. 7: Antibacterial activity of petroleum ether, ethyl acetate, chloroform, butanol, ethanol and water extracted samples from *Mentha longifolia* against *Erwinia carotovora* by disc diffusion assay.

Highest antibacterial activity against *P aeruginosa* was recorded by dichloromethane and ethyl acetate extracted samples (fig. 8).

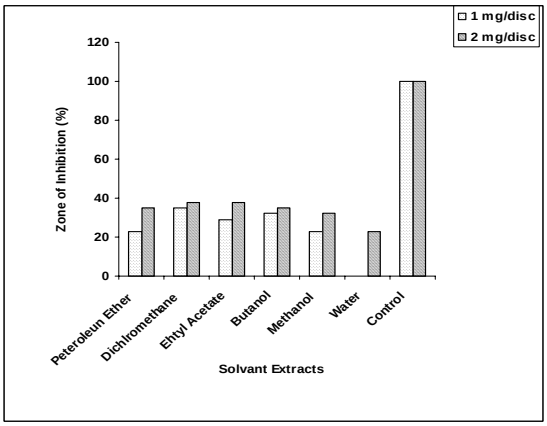

Fig. 8: Antibacterial activity of petroleum ether, ethyl acetate, chloroform, butanol, ethanol and water extracted samples from *Mentha longifolia* against *Pseudomonas aeruginosa* by disc diffusion assay.

At 2 mg disc^{-1}, both samples showed 38% zone of inhibition against *P aeruginosa*. Petroleum ether and butanol extracted samples were also effective in controlling the growth of *P aeruginosa* followed by

methanol extracted samples when compared with their positive controls. Water extracted samples, however, did not inhibit the growth at lower concentration of 1mg disc^{-1}, however, shown activity of 23% ZI 2 mg disc^{-1}. *A tumefacien* was susceptible to all extracts (fig. 9).

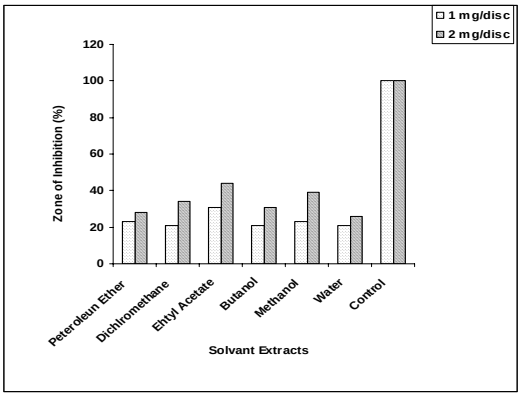

Fig. 9: Antibacterial activity of petroleum ether, ethyl acetate, chloroform, butanol, ethanol and water extracted samples from *Mentha longifolia* against *Agrobacterium tumefacien* by disc diffusion assay.

Maximum zone of inhibition was shown by ethyl acetate extracted samples recording 44% ZI at 2 mg disc^{-1} followed by methanol extracts showing 39% inhibition at 2 mg disc^{-1}. Dichloromethane and butanol extracted samples also showed reduction in the growth *A tumefacien* by 34% (1 mg disc^{-1}) and 31% at same concentration respectively. Petroleum ether and water extracted samples inhibited the growth of *A tumefacien* less efficiently.

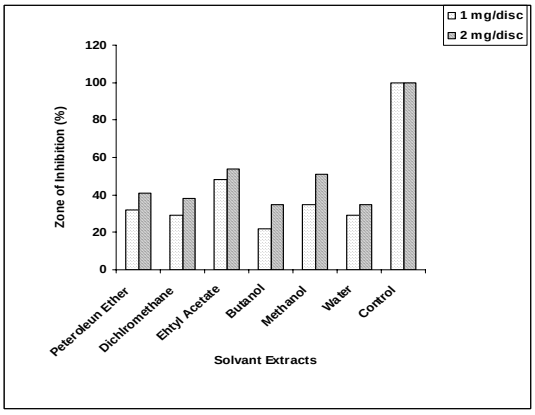

Fig. 10: Antibacterial activity of petroleum ether, ethyl acetate, chloroform, butanol, ethanol and water extracted samples from *Mentha longifolia* against *Klebsiella pneumoniae* by disc diffusion assay.

All extracts showed effective inhibitory activities against *Klebsiella pneumonia* when compared with their positive control ciprofloxacin. The susceptibility of bacteria increased with increasing concentration of the extracts. Highest activity was observed in case of ethyl acetate extracted samples and showed 48% and 54% zone of inhibition at 1 and 2 mg disc^{-1} concentration. Methanol fraction was also effective and showed 51% inhibitory activity at 2 mg disc^{-1}. This bacterium was also susceptible to petroleum ether, dichloromethane, butanol and water extracts and inhibited the growth of *Klebsiella pneumonia* by 34%, 38%, 35% and 35% respectively at high concentrations (fig. 10).

DISCUSSION

This present study investigate the antimicrobial activities of different solvents extracted samples from the aerial parts of *Mentha longifolia* against ten microbial species through the disc diffusion assay using two different concentrations. The data showed that *Bacillus subtilis* was susceptible to all extracts and revealed different ranges of antibacterial activities. Ethyl acetate fraction was more effective at higher concentration followed by dichloromethane at the same concentration. Water extracted samples were least effective in controlling the growth of this bacterium. Petroleum ether and butanol extracted samples were found to be equally effective against *B subtilis* at higher concentration (2 mg disc^{-1}). These results agree with Sokmen *et al.* (2000). Petroleum ether, butanol, dichloromethane and methanol extracted samples showed almost the same inhibitory activities against *S aureus* while dichloromethane extracted samples showed slightly higher activity against *S aureus* at 2 mg disc^{-1} concentration. Contrary to that water extracts was ineffective at lower concentration, however, inhibitory activity was noted at higher concentration. Ethyl acetate extracted samples were more effective to reduce the growth of *S aureus* compared with other solvent extracted samples. These results agree with Sokmen *et al.* (2000), Akroum *et al.* (2009) and Gulluce *et al.* (2007).

Petroleum ether extracts of the plant did not reduce the growth of *E coli* at lower concentration however at higher concentration some activity was measured. *E coli* were highly resistant to dichloromethane fractions and did not show activity at both concentrations. Ethyl acetate extracted samples were highly effective among all extracts to inhibit the growth of *E coli* at both concentrations. The antibacterial activity of butanol fraction was comparable at both concentrations. Similar results are also reported by Sokmen *et al.* (2000), Gulluce *et al.* (2007) and Akroum *et al.* (2009). *S typhi* was highly resistant to petroleum ether, dichloromethane and methanol extracted samples and did not show antibacterial activity against at both concentrations (0% ZI). Ethyl acetate, butanol and water fractions was found to be effective in controlling the growth of *S typhi* at both concentrations. The antifungal activity of different solvents extracted samples from *Mentha longifolia* against the *Candida albicans* revealed that ethyl acetate fraction showed more activity against *Candida albicans* at higher concentration compared with other fractions. *Candida albicans* showed resistance to low concentration of water

extracted samples, however, showed activity at higher concentration. The inhibitory effect of petroleum ether and butanol fraction was at par against the *Candida albicans*. Dichloromethane and methanol fractions were also effective against this fungus and both showed good activity at high concentrations.

Analysis of the data revealed that *E carotovora* was susceptible to all extracted samples and highest activity was recorded for petroleum ether fraction followed by dichloromethane, ethyl acetate, methanol and butanol samples at high concentration i.e. 2 mg $disc^{-1}$. However, water extracted fraction was be less effective against *E carotovora* when compared with other fractions. Antibacterial activity of different extracts from *Mentha longifolia* against *Bacillus atropheus* revealed that highest activity was observed in case of petroleum ether extracted samples followed by dichloromethane and ethyl acetate fractions. Butanol and water extracted samples showed similar inhibitory activity at high concentration. Highest antibacterial activity against *P aeruginosa* was recorded by dichloromethane and ethyl acetate extracted samples. Petroleum ether and butanol extracted samples were also effective in controlling the growth of *P aeruginosa* followed by methanol extracted samples when compared with their positive controls. Water extracted samples, however, did not inhibit the growth at lower concentration, however, shown activity at 2 mg $disc^{-1}$. These results agree with Sokmen *et al.* (2000). *A tumefacien* was susceptible to all extracts. This bacterium was also susceptible to petroleum ether, dichloromethane, butanol and water extracts both concentrations. Similar results are also reported by Mkaddem *et al.* (2009). Menthol ($C_{10}H_{20}O$) is a terpenoid, found in the essential oils of the mint family (*Mentha* spp.) such as peppermint, horse mint and others. Terpenes or terpenoids have been previously shown to be active against bacteria (Ahmad *et al*., 1993; Amaral *et al*., 1998), fungi (Harigan *et al*., 1993; Rana *et al*., 1997), viruses (Hasegawa *et al*., 1994; Xu *et al*., 1996) and protozoa (Vishwakarma, 1990; Ghoshal *et al*., 1996). The possible mechanism of action of terpenes is it not involvement in membrane disruption by the lipophilic compounds (Cowan, 1999).

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