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Full Length Research Paper

Impact of geographical locations on *Mentha spicata* antibacterial activities

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Medicinal plants produce secondary metabolites that are responsible for their therapeutic properties but the presence of these molecules and conversely their activities is affected by environmental factors like geographical location, fertility of cultivar, part used, season and time of collection. The present study is based on the impact of geographical location on the antibacterial activities of *Mentha spicata* L. The samples of the plant were collected from eight different locations of Khyber Paktoonkhwa (KPK) province of Pakistan, which included districts of Swat, Mardan, Charsada, Swabi, Peshawar, Kohat, Karak and Bannu. The samples were subjected to hydroalcoholic extraction followed by fractionation with n-hexane, chloroform, ethyl acetate and n-butanol in order of increasing polarity so as to separate the metabolites on the basis of their solubility in respective solvents. A broth macrodilution method was utilized to evaluate the minimum inhibitory concentrations (MIC's) of the samples against six bacteria (*Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Shigella flexenari*). The results of the study indicated *B. subtilis* and *S. aureus* to be the most sensitive bacteria (MIC = 0.06 – 0.125 mg/ml) while the Gram-negative bacteria were having moderate activities (MIC = 0.06 – 0.5 mg/ml). The samples obtained from the higher altitude regions (Swat, Swabi) and fertile cultivars (Charsada, Peshawar, Mardan) were found to be more effective as far as the antibacterial activities are concerned that signify a positive impact of geographical location and richness of land with respect to nutrients.

Key words: *Mentha spicata, Lamiaceae,* antibacterial activity.

INTRODUCTION

Plants may produce as much as 100,000 small-molecules (Dixon, 2001), which include primary metabolites that are present essentially in all plants resulting from primary metabolic activities, but the secondary metabolites that are specific to certain plant species, are produced in small quantities, have geographical impact on their production and are generally produced in a particular plant part (Zhi-linn et al., 2007). These compounds are the result of the secondary metabolic pathways that take place in certain plant species. For plants little effects have been attributed to these potential substances like, defense against microorganisms, insects and herbivores. While some of them give plants their odors and pigments many of them are responsible for plant flavors but for humans, at instances, they become life saving drugs (Evans, 2002). These metabolites have shown to possess antimicrobial

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Abbreviations: KPK, Khyber Paktoonkhwa; **DMSO,** dimethyl sulfoxide; **NA,** Nutrient agar; **NB,** nutrient broth; **MHA,** Muller Hinton agar; **MHB,** Muller Hinton broth; **MIC,** minimum inhibitory concentration.

potentials and their antimicrobial mechanisms include substrate deprivation, membrane disruption, binding to adhesins making them inactive, complexation with the cell wall, inactivation of the enzymes, and metal ion complexation leading to inactivation of such ligands that depend on those ions for functionality (Cowan, 1999).

Lamiaceae previously known as Labiatae, and also termed mint family consists of flowering plants. Its genus *Mentha* has some 20 species. *Mentha spicata* which is a perennial herb (Rana et al., 1997). It has been used in traditional medicines for number of indications like spasmolytic agent for colic and flatulence, as cholagogue, an anti-inflammatory and ulcer healing remedy (Mabey and Mclntyre, 1988) and also having antimicrobial potentials. The high concentrations of of terpenes, terpenoids (Soković et al., 2009) and also volatile oils, tannins, coumarins, flavonoids, steroids and alkaloids (Mabey and Mclntyre, 1988) justify the antimicrobial indications since these metabolites are related to antimicrobial activities (Cowan, 1999) that justifies the use of this plant in infections. With a more recent work by Padmini et al. (2010) that positively reports the antibacterial potential of this plant.

Geographical location can impact the quantity of secondary metabolites in plant species thereby, affecting the presumed activities that are related to a medicinal plant (Badoni et al., 2009; Gupta et al., 2011; Yang et al., 2004). In case of *Mentha* species, four spearmint, and two peppermint clonal lines, selected for enhanced rosmarinic acid content (50 to 120 mg g^{-1} rosmarinic acid), where up to 80% of the antioxidant activity was correlated to rosmarinic acid content, were examined to for the effects of environmental and physiological conditions on the accumulation of rosmarinic acid in leaf tissues. This study positively indicated the variation of content due to the environmental and physiological conditions; therefore, any geographical change that commonly ensues a change in environment will affect the content of secondary metabolites and conversely the presumed medicinal activity (Fletcher et al., 2010).

Keeping in view the available information it is evident that Mentha species posses' antimicrobial activities especially against pathogenic microorganisms. There is variation of phytochemicals that can be related to geographical location, seasons, and soil fertility that can ultimately affect the antimicrobial activities. Since there is limited information available on *M. spicata* as a whole and as far as Pakistan is concerned, therefore, the present study was designed to investigate and compare the antimicrobial activities of samples obtained from eight different geographical locations of Khyber Paktoonkhwa (KPK) for the plant *M. spicata*, which included Swat (984 m), Mardan (283 m), Charsada (276 m), Peshawar (510 m) and Swabi (521 m), representing the northern parts of KPK. While Kohat (489 m), Karak (582 m) and Bannu (371 m) are located in southern part of KPK (altitudes from sea level in brackets). The northern parts have mild to moderate temperatures and land rich in nutrients while southern regions have extreme temperatures and less fertile lands.

MATERIALS AND METHODS

Chemicals, media and equipment

The organic solvents used for extraction and fractionation included methanol, n-hexane, chloroform, ethyl acetate, n-butanol and dimethyl sulfoxide (DMSO) were from Merck, Germany. Nutrient agar (NA), nutrient broth (NB), Muller Hinton agar (MHA) and Muller Hinton broth (MHB) were of Oxoid, UK that were used for culturing, and susceptibility assays. Clarithromycin and ciprofloxacin (positive controls for the bacteria) were from Sigma-Aldrich, Germany.

Plant material

M. spicata L. belongs to Lamiaceae family. The leaves of the plant were collected from eight different locations (Swat, Peshawar, Charsada, Mardan, Swabi, Kohat, Bannu, Karak) from KPK, Pakistan, in March 2010 and identified by Associate Professor Dr. Muhammad Ibrar, plant taxonomist. A voucher specimen number MS-012, has been deposited in the herbarium of the University of Peshawar.

Preparation of extract

The shade dried leaves (weighing 0.5 kg) of *M. spicata* were pulverized to a fine powder. Methanol (80% *v/v*) was used for extraction. The crude hydroalcoholic extract *M. spicata* was first deprived off the alcohol, and then fractionated using *n*-hexane, dichloromethane, ethyl acetate, and n-butanol. All of the fractions so obtained were filtered twice using Whatman No. 1 filter paper sheets and concentrated under reduced pressure using Rotavapor R-200 at 30 ± 5℃. The collections obtained were stored at 4℃.

Preliminary phytochemical screening

The crude methanolic extract of the plant material was tested for various classes of natural products using standard qualitative methods (Nisar et al., 2010).

Antibacterial susceptibility Assays

Susceptibility of test bacteria (*Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Shigella flexenari, Salmonella typhi)* was carried out using broth macrodilution assay (Bisignano et al., 2000). A loop full of bacterial culture, previously grown to pure culture level on NA, was inoculated in the NB and incubated at 37 ± 1℃ for 1 h. The fresh broth (20 ml) was seeded with 0.25 ml of 24 h broth cultures and half fold serial dilution method was followed such that the test sample was dissolved in sterile normal saline with DMSO to obtain

Table 1. Quantities of Crude and subsequent fractions obtained from *M. spicata* extraction and fractionation.

Mass obtained in grams	DISTRICTS OF KPK							
	Swt	***Mdn***	***Chd***	***Swb***	***Pesh***	***Kt***	***Kk***	***Bann***
Crude extract	445	425	456	474	463	437	440	449
n-Hexane fraction	45	50	52	49	50	40	45	49
Chloroform fraction	75	70	72	65	70	77	78	70
Ethyl acetate fraction	110	100	102	115	95	100	98	100
n-Butanol fraction	80	85	90	95	98	82	80	85
Aqueous fraction	135	120	140	150	150	138	139	145

(Swt = Swat; Mdn = Mardan; Chd = Charsada; Swb = Swabi; Pesh = Peshawar; Kt = Kohat; Kk = Karak; Bann = Bannu).

10 mg/ml solution. A 0.4 ml solution of test material was added to 1.6 ml of seeded broth and this made the first dilution. One ml of this dilution was diluted further with 1 ml of the seeded broth to produce the second dilution, and the process was repeated until eight such dilutions were formed. A set of tubes containing only seeded broth was kept as control and normal saline with DMSO served as negative control. Clarithromycin and ciprofloxacin served as positive controls for gram-positive and gram-negative bacteria respectively in 15 and 25 µg/ml concentrations, respectively. After incubation for 24 h at 37 ± 1℃, the tube with no visible growth of bacteria was considered as the minimum inhibitory concentration (MIC) of test sample.

RESULTS

The selected plant was obtained as whole samples from the selected locations during one week span, keeping in view the environmental variables that may affect the composition of phytochemicals.

Extraction and fractionation

The 5 kg plant material of *M. spicata* after extraction with 80% (v/v) methanol yielded variable quantities (Table 1) of crude extracts, which upon subsequent fractionation resulted in corresponding quantities of fractions. The highest yield was from the samples obtained from Swabi (474 g), Peshawar (463 g) and Charsada (456 g) while for the rest of the regions it was below 450 g. This might be due to the fertility of the cultivar of these regions. In case of n-hexane fraction the highest yield was from Charsada 52 g (11.40%). For chloroform fraction the highest yield was observed from sample obtained from Karak 78 g (17.73%). The optimum yield for the ethyl acetate fraction came from Swabi 115 g (24.26%). The sample from Peshawar gave highest quantity for the n-butanol fraction 98 g (21.27%). The aqueous fraction that was the left over from all the fractionation stages surpassed in quantities from all the organic fractions and the highest amounts were seen in case of Swabi, 150 g (31.65%) and Peshawar, 150 g (32.40%). These results are indicative of the variation of contents from different geographical locations and the apparent factor in this case can be the nutrient rich lands of the respective locations that provided the elements needed for the production of the secondary metabolites.

Phytochemical analyses

The results of phytochemical screening indicated the presence of tannins, alkaloids, flavonoids, steroids, coumarins, terpenes and terpenoids while the tests for saponins and anthraquinone remained negative for all samples.

Antibacterial activity

The impact of the crude extracts and fractions thereof was overall quite good against the test bacteria. The Gram-positive showed more sensitivity in comparison to Gram-negative bacteria. The lowest MIC's observed in case of *B. subtilis* is from the sample obtained from Swat and Swabi (0.06 mg/ml). The test bacterium showed high sensitivity to the samples. Moreover the activities were confined in the ethyl acetate fractions of the samples indicating some useful moiety that is present in it. The pathogenic *S. aureus* also showed good susceptibilities with lowest ones (0.125 mg/ml) in the sample from Swat, Charsadda, Swabi. The activities were seen mostly in the crude extract and chloroform fraction for this bacterium.

Against *P. aeruginosa* lowest MIC was observed in the crude extracts from Swat and Kohat (0.06 mg/ml) and activities were generally confined in the crude extracts. This pattern was also the case for *S. flexenari*, where the lowest MIC observed was 0.06 mg/ml, from the sample obtained from Swat and Peshawar and most of the activities were seen in case of crude samples. Crude extract as well as chloroform fractions were also effective in the case of *E. coli* but the lowest MIC's were observed in chloroform fractions (0.125 mg/ml) from Swat and Kohat regions whereas crude fraction from Swabi was having the same MIC. *S. typhi* was only inhibited complete at 0.125 mg/ml by chloroform fraction from the sample originating from Bannu.

Table 2. Antibacterial activity, in terms of lowest MIC (mg/ml), of the crude extract and fractions of *Mentha spicata* from districts of KPK.

	DISTRICTS OF KPK							
Bacteria	***Swt***	***Mdn***	***Chd***	***Swb***	***Pesh***	***Kt***	***Kk***	***Bann***
Gram-positive								
B. subtilis	0.06 (Et)	0.125 (Et)	0.25 (Et)	0.06 (Et)	0.125 (Et)	0.125 (Et)	0.5 (Et)	0.125 (Et)
S. aureus	0.125 (Cl)	0.25 (Cr)	0.125 (Cl)	0.125 (Cl)	0.5 (Cl)	0.25 (Cl)	0.5 (Cr, Cl)	0.5 (Cl)
Gram-negative								
P. aeruginosa	0.06 (Cr)	0.125 (Cr)	0.5 (Cl)	0.125 (Cl)	0.5 (Cr)	0.06 (Cr)	0.5 (Cr)	0.5 (Cr)
S. flexenari	0.06 (Cr)	0.5 (Cr)	0.125 (Cr)	0.5 (Cr)	0.06 (Cl)	0.25 (Cr)	1.0 (Cr)	0.5 (Cr)
E. coli	0.125 (Cl)	0.25 (Cr)	0.25 (Cl)	0.125 (Cr)	2.0 (Cr)	0.125 (Cl)	1.0 (Cl)	0.25 (Cr, Cl)
S. typhi	0.25 (Cl)	0.5 (Cr)	0.5 (Cr)	0.5 (Cl)	0.25 (Cr, Cl)	0.5 (Cr)	2.0 (Cr, Cl)	0.125 (Cl)

(*Cr* = Crude extract; *Cl* = Chloroform fraction; *Et* = Ethyl acetate fraction).

The Gram-positive bacteria, in general, were affected most by the ethyl acetate fractions of different samples followed by the chloroform fraction. This behavior indicates some polarity present in the molecules that are causing the inhibition. The case of Gram-negative bacteria is different in which the crude extracts of various geographical origins exhibited inhibitions suggesting some synergestic interaction among the molecules for the control of these bacteria followed by the chloroform fractions. Aqueous and n-butanol fractions of all samples remained ineffective against the test bacteria.

The negative control used for the assays included normal saline and DMSO that had no inhibitory impact upon both types of test bacteria. The positive controls, clarithromycin for the Gram-positives and ciprofloxacin for the Gram-negatives, were effective (MIC's 0.0003 mg/ml) in their control, thereby suggesting them to be sensitive bacteria (Table 2).

DISCUSSION

M. spicata L. is commonly known as mint. It is used as flavors in food and preservative. It has shown to possess antibacterial activities mainly due to the essential oils against gram-positive and Gram-negative bacteria (Singh et al., 1994). It is having a number of phytochemicals especially the essential oils containing tricyclene, thujene, pinene, sabinene, pinene, myrcene, cymene, limonene, cineole, terpinene, menthone, menthol, terpin-4-ol, carvones, anethole, caryophyllene, germacrene that can be related to the antimicrobial activities (Soković et al., 2009).

Mentha spp. have shown to possess antimicrobial activities especially against bacteria and fungi (Singh et al., 1994). Their impacts have also been seen in bacteria like *H. pylori, S. enteritidis, E. coli,* MRSA, MSSA, *L. monocytogenes* and many other pathogenic bacteria (Tassou et al., 1995; Oumzil et al., 2002). Similarly, in cases of fungi it has shown activities against notable pathogen *A. flavus* (Marotti et al., 1994; Montes-Belmont and Carvajal, 1998), and *F. oxysporum* (Singh et al., 1994; Oumzil et al., 2002).

This study was carried using six bacteria including *P. aeruginosa,* which is an environmental inhabitant and an opportunistic pathogen (Forbes et al., 2007), *E. coli* which is involved in causing severe infections of the urinary tract (of both community and nosocomial origin), sepsis, meningitis, and diarrheal diseases (Brooks et al., 2004), *S. typhi* that is the causative agent for the enteric fevers, enterocolitis, and septicemia (Levinson, 2004), *S. flexenari* that cause Shigellosis (Torres, 2004), *S. aureus* is pathogenic Gram-positive cocci that is involved in infections of skin, soft tissue, respiratory tract, bone, joint, and endovascular disorders, (Lowy, 1998), and *B. subtilis* is a nonpathogenic bacterium (Brooks et al., 2004).

The lowest MIC observed in case of gram-positive bacteria was against *B. subtilis* while all the samples showed good impact against the test bacteria (MIC = 0.06 - 0.5 mg/ml) this can be well inferred by the presence of terpenes that show good activity against the bacteria (Cowan, 1999).

Since this plant is rich in terpenes (Soković et al., 2009) the sensitivity of bacterial strains is understandable. Since similar results have been reported against the gram-positive bacteria (Sivropoulou et al., 1995; Oumzil et al., 2002; Singh et al., 1994) this study confirms them with a variation effect report as far as geographical locations are concerned. Furthermore, the activities are mainly observed in the ethyl acetate, followed by chloroform fractions, which suggest the presence of phytochemicals that are responsible for inhibition in them. As far as geographical impact is concerned the samples from Swat and Swabi showed the lowest MIC's suggesting that the impact of higher altitudes and cool temperatures of the area on the presence of secondary metabolites causing inhibition.

The lowest MIC's observed in case of Gram-negative bacteria were for *P. aeruginosa* and *S. flexenari* (0.06 mg/ml) while all the samples showed a moderate impact

against the test bacteria (MIC = 0.06 to 2 mg/ml). This indicates that the test bacteria are rather hard to inhibit. Since there is difference in cell walls of gram-positive and gram-negative bacteria, therefore, multiplicity of components was required to check the growth of the test bacteria as is implicated by the majority of activities been shown by the crude extracts. This indicates also that a synergistic effect has caused the inhibition in general but there are certain fractions like chloroform and ethyl acetate that can be further searched for metabolites as they have also posed challenge to the bacteria. The inhibition of gram-negative bacteria is evident from other studies (Sivropoulou et al., 1995; Oumzil et al., 2002) as well that were conducted on *S. enteritidis, E. coli* and *L. monocytogenes* (Tassou et al., 1995). This study confirms their reports and also report activity against *P. aeruginosa* as well. From geographical location point of view, samples from Swat topped the list followed by Mardan, Charsadda and Kohat, while the samples from the districts located at low altitudes were having less impact on the control of gram-negative bacteria.

All of the test samples showed considerable antibacterial activity against the test bacteria. The effects can be attributed to the presence of essential oils in *M. spicata* that is enriched with terpenes and terpenoids (Soković et al., 2009), which have got the potential to inhibit microorganisms (Cowan, 1999), therefore, justifying the inhibitions caused by the fractions and crude extracts of this plant.

Generally the samples originating from the higher altitudes especially from Swat and Swabi were shown to possess more activity than the samples from the plain areas. However, samples from Charsada and Peshawar also gave good results that can be related to fertility of land and a medium altitude from sea level that can have a role in metabolites production. Altitudinal variation in plant constituents exist and same can be the case of *Mentha* spp. Our study confirms a difference in activity that can be related to the variation in phytochemical content and also to the geographical location. This is particularly true, since in the previous studies it was found that the essential oil content varies due to alteration in geographical locations mainly because of the temperature effect that is detrimental in case of the volatile components that may have a significant role to play in the antimicrobial activity and also to land fertility that can also be a factor involved in the presence of secondary metabolites and their presumed activities.

Conclusion

The results of the study quantitatively express the impact of geographical locations on the presence of secondary metabolites responsible for antibacterial activities. They also indicate the effect of cultivar fertility on their presence that can be further searched with more detailed analyses of the soil and metabolites content. The overall impact can be utilized in the selection of suitable regions for the cultivation of this herb and furthermore, similar studies can be designed for other medicinal herbs to outline the environment and location that can maximize their yields.

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