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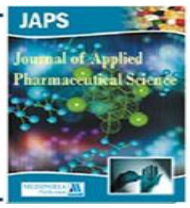

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Comparison of Phytochemical constituents and antimicrobial activities of *Mentha spicata* from four northern districts of Khyber pakhtunkhwa

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ABSTRACT

The medicinal value of plants lies in bioactive phytochemical constituents that produce definite physiological actions on the human body. Some of the most important bioactive phytochemical constituents are Tannin, Alkaloids, Saponins, Flavonoids, Steroids, Anthraquinones, Coumarins and Sterols and Terpenes. Infectious diseases are the leading cause of death worldwide, accounting for nearly one half of all deaths in tropical countries which are also becoming a significant problem in developed countries. Therefore the present study was aimed to analyzed the phytochemicals and antimicrobial activities of *Mentha spicata*. The phytochemical constituents were determined by using known literature method while the antimicrobial activity was analyzed by classical literature methods. In case of phytochemicals, Tannin, Alkaloids, Glycosides, Flavonoids, Steroids, Coumarines, Sterols and Terpenes were found while saponins and anthraquinones were not determined in all the samples. Antibacterial activity was noted high in all the samples of crude extract followed by ethyl acetate and lowest activity was found was found in aqueous extract of *Mentha spicata*. The Same results were analyzed for antifungal activity.

Key words: *Mentha spicata*, Lamiaceae, Antifungal activity, Antibacterial activity, Khyber Pakhtunkhwa.

INTRODUCTION

The term herb refers to a plant used for medicinal purposes. To the uninformed, herbs are generally thought of as ineffective medicines used prior to the advent of more effective synthetic drugs. To others, herbs are simply sources of compounds to isolate and then market as drugs. But to some, herbs and crude plant extracts are effective medicines to be respected and appreciated. For many people herbal medicines are the only therapeutic agents available. In 1985, the WHO estimated that perhaps 80%of the world population relies on herbs for primary health care needs (Farnsworth et al., 1985). This widespread use of herbal medicines is not restricted to developing countries, as it has been estimated that 30-40% of all medical doctors in France and Germany rely on herbal preparations as their primary medicines. The world is rich in natural and unique medicinal plants. Medicinal plants are now getting more attention than ever because they have potential of large benefits to society or indeed to all mankind, especially in the line of medicine. The medicinal value of these plants lies in bioactive phytochemical constituents that produce definite physiological actions on the human body. Some of the most important bioactive phytochemical constituents are Tannin, Alkaloids, Saponins, Flavonoids, Steroids, Anthraquinones, Coumarins and Sterols and Terpenes. Clinical microbiologists have two reasons

to be interested in the topic of antimicrobial plant extracts. First, it is very likely that these phytochemicals will find their way into the arsenal of antimicrobial drugs prescribed by physicians; several are already being tested in humans. It is reported that, on average, two or three antibiotics derived from microorganisms are launched each year (Casley-Smith, 1997). After a downturn in that pace in recent decades, the pace is again quickening as scientists realize that the effective life span of any antibiotic is limited. Worldwide spending on finding new anti-infective agents (including vaccines) is expected to increase 60% from the spending levels in 1993 (Alper, 1998). New sources, especially plant sources, are also being investigated. Second, the public is becoming increasingly aware of problems with the over prescription and misuse of traditional antibiotics. *Mentha spicata* is a perennial herb commonly known as mint, belong to the family Lamiaceae. It is a glabrous herb identical to pippermint,30-90 cm n height, but the stem are usually more purple in color with variably hairless to hairy stems and foliage, and a wide-spreading fleshy underground rhizome. Leaves are more or less crumpled, opposite, ovate-lanceolate,3-7 cm long. The apex of leaf is acute or acuminate, and has unequally serrate margin. The leaves are almost sessile with bright green color free from purple. Inflorescence is slender, interrupted cylindrical spikes are crowded lanceolate spikes with 7-10 cm long bracts. *M. spicata* contain volatile oils, resins, tannins. Coumarins Flavonoids. Steroids. Alkaloids. The principle constituent is carvone along with some other monoturpenic constituents like limonene, phellendrene, dihydrocarveol, dihydrocarveol, cetate, cineol, alpha pinene and linalool. It possesses antiemetic properties also used as analgesic, stimulant, expectorant and carminative, digestion/ingestion. It can also acts as Refrigerant, Analgesic, Aphrodisiac, stimulant, Expectorant and anti-inflammatory. Antiseptic; Antispasmodic, and Diuretic, Spearmint has been studied for antifungal activity; its essential oil was found to have some antifungal activity (Sweetie et al., 2007). Keeping in view the medicinal importance of this plant the present study was aimed to investigate the phytochemicals and antimicrobial activity from the four northern districts of Khyber Pakhtunkhwa.

MATERIALS AND METHODS

Extraction and Isolation

Simple extraction procedure was adopted for *Mentha spicata* Whole plant material dried under shade was chopped and pulverized into fine powder. 5.0 kg of dried powder was macerated with 80 % methanol three times at room temperature. Resulting methanolic extract (479.31 g) was evaporated under vacuum by rotary evaporator at 45 °C that afforded a gummy residue. The crude extract (470 g) was suspended in water and fractionated successively with n-hexane, chloroform and ethyl acetate, followed by leaving a residual water-soluble fraction. Each fraction was then concentrated using rotary evaporator at 50°C to yield n-hexane fraction (81.69g, 17.38%), chloroform fraction (108.97 g, 23.19%) and ethyl acetate fraction (79.14 g, 16.84%), the remaining was water fraction (196.82 g, 41.88%).(A)

Phytochemical Analysis

The crude methanolic extract of the plant material was tested for various classes of natural products using standard qualitative methods. Briefly the methods are given below and results are summarized in table 1.

Tannins

The test for tannins was performed by subjecting 1 g plant extract in 2 ml distilled water, filtered and ferric chloride reagent was added to the filtrate.

Alkaloids

For alkaloids, the test was carried out by subjecting 1 g methanolic extract in 10 ml 1% HCl, boiled, filtered and Mayer's reagent was applied.

Saponins

The extract was subjected to frothing test for the identification of saponins.

Flavonoids

The presence of flavonoids was determined by using 1% aluminum chloride solution in methanol concentrated HCl, magnesium turnings, and potassium hydroxide solution.

Steroids

Steroids were screened by adding 1 ml of acetic anhydride to 0.25 g methanolic extract of each sample with 1 ml H_2SO_4. The color changed from violet to blue or green in some samples indicating the presence of steroids.

Anthraquinones

The test for anthraquinones was performed by adding 1 g of extract to 2ml benzene, filtered and ammonia solution added.

Coumarins

For detecting coumarins, a piece of filter paper was moistened in NaOH and then kept over a test tube with boiling plant extract solution. If the filter paper later showed any yellow fluorescence under UV light that was taken to indicate a positive test for coumarins.

Terpenes

Detection for any sterols and terpenes in the extract involved treatment of the extract with petroleum ether followed by extraction with $CHCl_3$. The subsequently acquired $CHCl_3$ layer was treated with acetic anhydride and concentrated HCl. The change of pink to purple and green to pink colors was indicative of the presence of terpenes or sterols, respectively (Nisar et al., 2010).

ANTIMICROBIAL ACTIVITY

Agar Diffusion Method

Ten ml aliquots of nutrient broth is inoculated with the test organism and incubated at 37°C for 24 hours. Using sterile pipette, 0.6ml of the broth culture of test organisms added to 60ml

of molten agar which has been cooled to 45c, mixed well and poured into a sterile Petri dish. For the 9cm Petri dish, 0.2ml of culture is added to 20ml of agar. Duplicate plates of each organism are prepared. The agar is allowed to set and harden and required no of holes are cut using sterile cork borer ensuring proper distribution of holes (cups) in the periphery and one in the centre. Agar plugs are removed. Different cork borer should be used for different test organisms. Using 0.1ml pipette,100µl of test sample dissolved in an appropriate solvent is poured into appropriately labeled cups(these are marked at the back of cops before filling).The same concentration of standard antimicrobial agents(streptomycin 1mg/ml and ampicillin10µg/ml) and the solvent (as control) are used. The plates are left at room temperature for 2 hours .to allow the diffusion of sample and incubated face upwards at 37c for 24 hours. The diameter of zone of inhibition is measured to nearest mm (the cup size also being noted).

Antibacterial Assay

A loop full of bacterial culture is inoculated in the nutrient broth and incubated at $37^{o}\pm 1^{o}C$ for one hour. The fresh broth (20ml) is seeded with 0.25ml of 24 hours broth cultures and two folded serial dilution methods is followed as described below. The test sample is dissolved in water (incase of water soluble sample) or in organic solvents (ethanol and acetone) to obtain 10mg/ml solution. A 0.2ml solution of test material is added to 1.8ml of seeded broth and this forms the first dilution. One ml of this dilution is diluted further with 1ml of the seeded broth to produce the second dilution, and the process is repeated until six such dilutions are formed. A set of tubes containing only seeded broth is kept as control and suitable solvents control are also maintained. After incubation for 24 hours at $37^{o}\pm 1^{o}C$ the last tube with no visible growth of microorganism is taken to represent the minimum inhibitory concentration (MIC) of test sample which is expressed in mg/ml (Hufford et al. 1993; Batista et al. 1994)

Antifungal Assay

Test sample was dissolved in sterile DMSO to serve as stock solution. Sabouraud dextrose agar is prepared by mixing Sabouraudf 4% glucose agar and agar in distilled water. It is then stirred with the magnetic stirrer to dissolve it and a known amount is dispensed into screw cap test tubes. Test tube containing the media is autoclaved at $121^{0}C$ for 15 minutes. Tubes were allowed cooling to $50^{o}C$ and the test sample of desired concentration pipette from the stalk solution into the no solidified Saboouraud agar media. Tubes are than allowed to solidify in slanting position at the room temperature. Each tube is inoculated with 4mm diameter piece of inoculums removed from 7 days old culture of fungi. All the culture containing tubes are inoculated at optimum temperature of $28\text{-}30^{o}C$ for growth for 7-10 days. Humidity (40%to 50%) is controlled by placing an open pan of water in incubator. Culture is examined at least twice weekly during the incubation. After incubation from seven to 10 days, the test tube with no visible growth of microorganism is taken to represent the minimum inhibitory concentration (MIC) of the test sample which is expressed in µg/ml.(Zhang and Lewis, 1997; Rana et al., 1997)

RESULT AND DISCUSSION

Phytochemicals

The world is rich in natural and unique medicinal plants. Medicinal plants are now getting more attention than ever because they have potential of large benefits to society or indeed to all mankind, especially in the line of medicine. The medicinal value of these plants lies in bioactive phytochemical constituents that produce definite physiological actions on the human body. Some of the most important bioactive phytochemical constituents are Tannin, Alkaloids, Saponins, Flavounoids, Steroids, Anthraquinones, Coumarins and Sterols and Terpenes (Hamburger and Hotesttmann, 1991). Phytochemicals study were carried out for qualitative analysis of Tannin, Alkaloids, Flavounoids, Steroids, Cumarines,Sterols and Terpines which were identified in all district including Malakand, Mardan, Charsada, Swabi While saponins and anthraquinones were not determined as shown in table 1.

Table 1: Qualitative Analysis of Phytochemicals.

Phytochemical	Analysis			
	Mkd	Mdn	Chd	Swb
Tannins	+	+	+	+
Alkaloids	+	+	+	+
Saponins	-	-	-	-
Flavonoids	+	+	+	+
Steroids	+	+	+	+
Anthraquinones	-	-	-	-
Coumarins	+	+	+	+
Sterols and Terpenes	+	+	+	+

Antibacterial Activity

Infectious diseases are the leading cause of death worldwide, accounting for nearly one half of all deaths in tropical countries which are also becoming a significant problem in developed countries. It is calculated that infectious diseases are the underlined causes of death in 8% of the 9 deaths in the united state. Development of new antimicrobial agents is among the proposed solution to solve this problem. In this regard plants can be provided a good alternative in search for new chemicals with a wide range of antibacterial and antifungal activities (Casley-Smith, 1997).

As can be seen in table 2 all of the test samples showed considerable antibacterial activity against the bacterial strains used in the study. As a whole, crude extract was found to be the most active extract. However in case of fractions, chloroform fraction was found to be the second most active sample followed by ethyl acetate, butanol and hexane fraction. Table 2 data shows that the crude extract was the most active extract, while the ethanol fraction was the second most active sample followed by chloroform, butanol and hexane fraction. Aqueous fraction showed the lowest activity among all samples.

All of the test samples showed considerable antibacterial activity against the bacterial strains used in the study. As a whole, crude extract was found to be the most active extract. In case of

Table 2: Antibacterial activity of the extract and fractions of *Mentha spicata* collected from Malakand in terms of Minimum Inhibitory Concentration (MIC, mg/mL).

Microorganism	Minimum Inhibitory Concentration (MIC, mg/mL)						
	cipro	Cr	Hx	Cl	Et	Bu	Aq
Escherchia .coli	0.0002	0.50	-	0.5	0.75	2.0	-
Bacillus subtilis	0.0005	0.50	2.0	0.75	0.25	0.75	-
Shigella flexeneri	0.0003	0.75	2.25	1.75	0.75	2.0	0.75
Staphylococcus aureus	0.0009	0.25	-	0.12	0.25	1.75	1.75
Pseudomonas aeruginosa	0.0021	0.25	1.75	0.5	0.5	2.25	0.75
Salmonella typhi	0.0014	0.75	2.0	0.5	0.75	2.0	-

Cipro:ciprofloxacin, Cr: Crude extract, Hx: n-hexane, Cl:Chloroform, Et: Ethyle acetate, Bu:n-butanol, Aq: Aqueous

Table 3: Antibacterial activity of the extract and fractions of *Mentha spicata* collected from Mardan in terms of Minimum Inhibitory Concentration (MIC, mg/mL).

Microorganism	Minimum Inhibitory Concentration (MIC, mg/mL)						
	cipro	Cr	Hx	Cl	Et	Bu	Aq
Escherchia .coli	0.0002	0.25	-	0.75	0.75	1.75	-
Bacillus subtilis	0.0005	0.25	1.75	0.5	0.12	0.75	-
Shigella flexeneri	0.0003	0.50	2.0	2.0	0.75	2.0	1.75
Staphylococcus aureus	0.0009	0.25	-	0.25	0.5	1.75	1.75
Pseudomonas aeruginosa	0.0021	0.12	0.75	0.25	0.25	2.0	0.5
Salmonella typhi	0.0014	0.5	0.75	0.75	1.75	1.75	-

Cipro:ciprofloxacin, Cr: Crude extract, Hx: n-hexane, Cl:Chloroform, Et: Ethyle acetate, Bu:n-butanol, Aq: Aqueous.

Table 4: Antibacterial activity of the extract and fractions of *Mentha spicata* collected from charsada in terms of Minimum Inhibitory Concentration (MIC, mg/mL).

Microorganism	Minimum Inhibitory Concentration (MIC, mg/mL)						
	cipro	Cr	Hx	Cl	Et	Bu	Aq
Escherchia .coli	0.0002	0.25	-	0.25	1.75	0.75	-
Bacillus subtilis	0.0005	1.75	1.75	0.75	0.25	0.5	-
Shigella flexeneri	0.0003	0.12	2.0	2.0	1.75	2.0	1.75
Staphylococcus aureus	0.0009	0.5	-	0.06	0.75	0.75	0.75
Pseudomonas aeruginosa	0.0021	0.75	0.75	0.5	0.75	1.75	1.75
Salmonella typhi	0.0014	0.75	0.75	0.75	0.5	2.0	-

Cipro:ciprofloxacin, Cr: Crude extract, Hx: n-hexane, Cl:Chloroform, Et: Ethyle acetate, Bu:n-butanol, Aq: Aqueous

Table 5: Antibacterial activity of the extract and fractions of *Mentha spicata* collected from Swabi in terms of Minimum Inhibitory Concentration (MIC, mg/mL).

Microorganism	Minimum Inhibitory Concentration (MIC, mg/mL)						
	cipro	Cr	Hx	Cl	Et	Bu	Aq
Escherchia .coli	0.0002	0.12	-	1.75	1.75	0.75	-
Bacillus subtilis	0.0005	0.25	0.75	0.75	0.06	0.5	-
Shigella flexeneri	0.0003	0.5	2.0	0.75	0.12	1.75	0.75
Staphylococcus aureus	0.0009	0.5	-	0.25	0.12	0.75	0.5
Pseudomonas aeruginosa	0.0021	0.25	1.75	0.12	0.75	2.25	0.75
Salmonella typhi	0.0014	0.75	0.75	0.5	0.75	1.75	-

Cipro:ciprofloxacin, Cr: Crude extract, Hx: n-hexane, Cl:Chloroform, Et: Ethyle acetate, Bu:n-butanol, Aq: Aqueous.

Table 6: Antifungal activity of the extract and fractions of *Mentha spicata* collected from Malakand in terms of zone of inhibition (mm).

Microorganism	Minimum Inhibitory Concentration (MIC, mg/mL)						
	clot	Cr	Hx	Cl	Et	Bu	Aq
Trichophyton longifusus	0.0014	0.5	-	2.75	0.75	2.25	2.75
Microsporum canis	0.0006	2.25	-	3.5	2.25	2.75	-
Candida albicans	0.0001	0.75	3.5	3.5	0.5	3.5	3.5
Aspergillus flavus	0.027	1.75	4.0	2.75	0.75	3.5	2.25
Fusarium solani	0.0011	2.75	-	3.5	4.0	-	3.5
Candida glaberata	0.0003	1.75	-	2.75	1.75	-	-

Clot:clotrimazole, Cr: Crude extract, Hx: n-hexane, Cl:Chloroform, Et: Ethyle acetate, Bu:n-butanol, Aq: Aqueous.

Table 7: Antifungal activity of the extract and fractions of *Mentha spicata* collected from Mardan in terms of zone of inhibition (mm).

Microorganism	Minimum Inhibitory Concentration (MIC, mg/mL)						
	clot	Cr	Hx	Cl	Et	Bu	Aq
Trichophyton longifusus	0.0014	0.25	-	2.25	1.75	1.75	2.75
Microsporum canis	0.0006	2.75	-	4.0	1.75	2.75	-
Candida albicans	0.0001	0.5	2.75	2.75	0.5	4.0	2.75
Aspergillus flavus	0.027	2.25	3.5	2.75	0.75	2.75	2.75
Fusarium solani	0.0011	2.25	-	3.5	3.5	-	1.75
Candida glaberata	0.0003	0.75	-	2.75	2.25	-	-

Clot:clotrimazole Cr: Crude extract, Hx: n-hexane, Cl:Chloroform, Et: Ethyle acetate, Bu:n-butanol, Aq: Aqueous.

Table 8: Antifungal activity of the extract and fractions of *Mentha spicata* collected from Charsada in terms of zone of inhibition (mm).

Microorganism	Minimum Inhibitory Concentration (MIC, mg/mL)						
	clot	Cr	Hx	Cl	Et	Bu	Aq
Trichophyton longifusus	0.0014	0.25	-	2.75	0.5	2.25	2.25
Microsporum canis	0.0006	2.25	-	3.5	1.75	3.5	-
Candida albicans	0.0001	0.5	2.75	2.25	1.75	2.75	3.5
Aspergillus flavus	0.027	1.75	3.5	3.5	0.75	3.5	2.75
Fusarium solani	0.0011	2.25	-	2.25	3.5	-	3.5
Candida glaberata	0.0003	0.5	-	3.5	2.25	-	-

Clot:clotrimoxazole, Cr: Crude extract, Hx: n-hexane, Cl:Chloroform, Et: Ethyle acetate, Bu:n-butanol, Aq: Aqueous

Table 9: Antifungal activity of the extract and fractions of *Mentha spicata* collected from Swabi in terms of zone of inhibition (mm).

Microorganism	Minimum Inhibitory Concentration (MIC, mg/mL)						
	clot	Cr	Hx	Cl	Et	Bu	Aq
Trichophyton longifusus	0.0014	1.75	-	2.75	0.5	0.75	3.5
Microsporum canis	0.0006	2.25	-	3.50	1.75	2.75	-
Candida albicans	0.0001	0.25	2.25	2.75	1.75	3.5	2.25
Aspergillus flavus	0.027	0.75	4.00	1.75	0.5	2.25	3.5
Fusarium solani	0.0011	1.75	-	1.75	3.5	-	2.75
Candida glaberata	0.0003	1.75	-	2.25	2.25	-	-

Clot:clotrimoxazole, Cr: Crude extract, Hx: n-hexane, Cl:Chloroform, Et: Ethyle acetate, Bu:n-butanol, Aq: Aqueous

fractions, chloroform fraction was the second followed by ethanol and butanol fraction. As can be seen from table 5 the crude extract shows higher activity against all bacterial strains and in case of

fractions, chloroform fraction was the second most active sample. The activity showed by other fractions are in decending order ethyl acetate, butanol and hexane fraction, while the the lowest activity was found in aqueous fraction. The crude extract shows the highest antifungal activity and ethanol fraction was the second most active sample. The antifungal activity of other fractions was followed by ethyl acetate, butanol and aqueous fraction respectively. The lowest activity was determined in a hexane fraction. As can be seen from table 7 all of the test samples showed considerable antifungal activity against the fungal strains used in the study. As a whole, crude showed maximum activity followed by ethanol, aqueous and chloroform fraction respective order. The lowest activity was recorded in hexane fraction. All of the test samples showed considerable antifungal activity against the fungal strains. The crude extract shows high est activity extract. While the ethanol fraction was the second most active sample, followed by chloroform and aqueous fraction. Hexane fraction revealed the lowest activity among all samples. Table 9 data showed that all the samples show antifungal activity. As a whole, crude extract was found to be the most active extract and in case of fractions, ethanol fraction was the second most active sample followed by chloroform and ethanol fraction, while the lowest activity was noted in hexane fraction.

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