CHEMICAL ANALYSIS AND BIOACTIVITIES OF ESSENTIAL OILS OF SELECTED *MENTHA SPECIES* FROM LOWER HIMALAYA REGION OF PAKISTAN

Uzma Khan[1 *] , Maryam Shahid[2], Uzma Abdullah [2], Warda Ahamd [2] and M. Gulfraz [2]

Corresponding author; uzmakhan12692@yahoo.com

ABSTRACT

Plants secondary metabolites are responsible for various biological activities. Essential oils of plants are also secondary metabolites that have curative and pharmaceutical effects such as antibacterial, antifungal, antiviral and insect repellent. The *mentha* species are potential candidates for development of drugs due to their efficient antimicrobial, antioxidant and radical-scavenging activities and reliable cytotoxicity

In the current study *Mentha species* (*Mentha arvensis, M. longifolia* and *M. spicata)* were assessed for their phytochemicals. The essential oils contents by using gas chromatography mass spectroscopy (GC-MS) and FT-IR . The antioxidant, antibacterial, cytotoxic and mosquito repellent activities of plant extracts were also determined It was observed that various mentha extracts are good source of flavonoids, phenolic, tannins and saponins . All mentha specie contained higher quantity of essential oils. The higher antioxidant activity of DPPH assay was found as compared to ABTS and H2O2. Where as higher zone of inhibition was obtained for S aures as compared to other microorganism . Where as plant extracts have shown lower cytotoxicity . It is expected that due to out come of this study raw materials of mentha spp will get industrial application and might be used for preparation of drugs required for human health

Keywords; Phytochemicals , Chemical analysis, Bioactivities**,**

1.Comsats university campus Abbottabad

2. UIBB- Arid Agriculture University Rawalpindi

INTRODUCTION

About 80% of world rural populations rely on herbal remedies for the treatment of various human ailments. Medicinal plants have got the vital role in allopathic drugs, large number of the modern drugs such as digoxin, morphine, codeine, aspirin, vinblastine, cocaine, emetine, ephedrine, vinocristine, pilocarpine etc., have been isolated from plants (Abbasi *et al*., 2015).. After the more advancement of synthetic chemistry during the later part of 20th century over natural products, again the interest of pharmaceutical industry in medicinal plants reawaken and they look at source of herbal plants as chemical scaffolds for synthesis of drugs. The natural compounds from medicinal plants are safer and could be found to replace the synthetic drugs which constitutes about 70% of our drugs (Adedapo *et al*., 2009).

It was estimated by WHO that world market for drugs originated from plants, may account about Rs. 200 million. Among 2,50,000 plant species that belong to higher plant on land, more than 80,000 are playing a vital role in health care. The drugs were obtained by whole plant or from single part such as stem, leaves, flower, root, bark, seed, etc. Some were derived from excretory product such as resins, latex and gum. Allopathic medicines have adopted a large number of plant derived drugs. These constitutes are an important part of the modern pharmacopoeia (Ashafa *et al*., 2010). Some plants also contained the important chemical intermediates which are very essential for the manufacturing of modern drugs (solasodine, diosgenin and betaionine) (Agor and Ngogan, 2005).

Pharmaceuticals available in modern medicine are directly or indirectly derived from natural sources, which are also of great importance in the process of drug discovery due to their huge diversity in nature that permits the identification of lead molecules of much interest for the development of new therapeutic agents for pathological processes. It is believed that about 80% of world's total population use plants as a fundamental source of medicine (Beauchamp and Fridovic. 1971; Cefarelli *et al*., 2006). Modern science and technology plays an important role in approaching for cultivation, conservation and preservation of the herbal drug species through plants tissue culturing and plant molecular biology. The isolation of active constituents and their development into new therapeutics; standardization and other related aspects need to be focused for further development(Dillard and German, 200). The plants of the genus Phyllanthus (Euphorbiaceae) are widely distributed in most tropical and subtropical countries, and have long been used in folk medicine to treat kidney and urinary bladder disturbances, intestinal infections, diabetes, and hepatitis B (Dufour *et al*., 2000).

In the past three decades researchers on plants have revealed that plants have such constituents which have been used for cure and prevention of diseases (Farnsworth and Morris (1976). Plants have molecules which have potential for new drugs discovery and for the treatment of diseases (Feng *et al*., 2011).

Infectious diseases caused by bacteria are also cured by most of the medicinal plants. These plants are locally available, inexpensive and becoming more popular day by day.Metabolic syndrome causes incompatibilities in the body. Medicinal plants having large excess of phytochemicals were used for treatment of metabolic syndrome. An antibacterial activity have been observed from carbazole alkaloid "clausenol" purified from an alcoholic extract of the stem bark of clausena anisata. The alcoholic and acetonic extract has shown significant in-vitro antibacterial activity from the leaves of Cassia alata (Foster and Duke, 2000). Cytotoxic activity had been shown by plants such as Thevetia ahouai, Physalis viscosa, Piper jacquemontianum and Piper barbatum., Senna occidentalis, Tovomita longifolia and Lippia cardiostegia., Blepharocalyx salicifolius and Senna occidentalis against one cell line, SF-268 or MCF-7, respectively at GI_{50} values $\leq$10 µg/ml (Fowler, 2006) . Cancer is responsible for many deaths worldwide. For treatment of cancer, methanolic extracts of Croton membranaceus roots and Zanthoxylum xanthoxyloides bark showed markedly anti-proliferative activities against three human cancer cell lines such as DLD-1, MCF-7 and M14, using the MTT assay .Anti HIV 1, anti-malarial and anti-fungal activity *in-vitro* was shown by four compounds those were purified from an extract prepared from the fruit rind of Terminalia belerica viz termilignan, thannilignan, 7-hydroxy 3, 4 (Methylenedioxy) flavone and anolignan B (Graf *et al*., 2010). **The** antimalarial activities were tested in species like Japonica, *Artemisia maritimia* and *Artemisia nilegarica* in their ethanolic and petroleum extracts both *in vivo* and *in vitro*. The activities were conceded out *in vivo* by using Rane test in Balb/c which showed that all composites extended the endurance time of mice .Plants are equipped with many chemical constituents. These chemical constituents are classified into two types of metabolites i-e primary and secondary metabolites (Gulfraz *et al*., 2008) .

Primary metabolites are involved in the primary metabolism of plants. Some of the primary metabolites are nucleic acids, carbohydrates, lipids, proteins and chlorophyll. Plants store the excess of primary metabolites are found either in stem, leaves or roots, which are used as food in

our daily life (Hussain *et al*., 2014) .
Secondary metabolites include chemical constituents which do not get metabolized in primary metabolism. In the past they were regarded as excretory products or end products. Now their importance has been realized by the medicine. These constituents are now regarded as medicinally important constituents. Secondary metabolites play an important role of defense for the plants. They protect the plant from insects and also keep the herbivores away from them (Koehn and Carter, 2005).

The plant secondary metabolites could be classified into some major classes such as alkaloids, terpenoids and phenolics etc.Alkaloids are heterocyclic nitrogen containing compounds. They contain nitrogen, they are basic in nature and are alkali like compounds and have bitter taste. These heterocyclics are pharmacologically active compounds. On the basis of number of nitrogen present in the molecule of alkaloids, they are further divided into different structures like Atropine, containing single nitrogen and Ergotamine, containing penta nitogen **.**Alkaloids are crystalline in nature and contain oxygen e.g. Nicotine. Normally alkaloids are colorless but inn few cases they are colored for example serpentine, it is brownish red in color. Alkaloids are soluble in ether, chloroform and other non-polar solvents and divided into three classes (Krymow, 2002).

Terpenoids are used for medicinal purposes as well as for dietary purposes Terpenoids are used to flavor the food and drinks. These are also used as flavoring agents in toothpastes and cigarettes (Males and Farnsworth,1995). Wound healing and anti-microbial activities of terpenoids are reported. These types of terpenoids are present in the resins obtained from the plants. Phenolics are made up of hydroxyl group containing aromatic ring. There may be one or more hydroxyl group .Tannins, lignin polyphenolics and melanin are the polymeric phenolic compounds of plants which are medicinally important. In phenolic compounds, mostly sugar moieties are present therefore they are soluble in water (Gulfraz *et al*., 2008). In human diet flavonoids and polyphenolic compounds are commonly found and these are class of secondary metabolites. Tannins and Saponins are also a type of flavonoids. There is a close relationship between medicinal plants and drug discovery. Use of medicinal plants for treatment of diseases by humans has long history. Isolation of active compounds from medicinal plants led to the foundation of drug discovery. Morphine was isolated in 1817 by Serturner (pharmacist) from Opium. Isolation of caffeine, atropine, codeine was carried out between 1820-1850. German scientist discovered first synthetic drug and was named as Antipyrin (Newman and Cragg,2012). Pakistan has been bestowed with unlimited natural resources of medicinal plants. In Pakistan there are 25 medicine companies producing tibbi medicines on commercial scale. Export Promotion Bureau of Pakistan reported that in 1999, Pakistan exported 8500 tons of medicinal

plant materials and yet earned 6 million US dollars as compared to 31 million dollars spent on import of herbal raw materials from other countries (Newman *et al*., 2000; Rios, 2010) .Almost 50,000 herbalists are working in different areas of Pakistan. 2000 species of Pakistani plants are given position in Unani system but only 400-600 have been documented. Pakistan pharmaceutical industries depends on medicinal plants for production of drugs and local practitioners uses these medicinal plants for cure of human ailments (Packer *et al*., 2004). Similarly 70 species of medicinal plants (Pandey,2006). In Pakistan, a large variety of lamiaceae flora is inhabited consisting of total 91 species of family lamiacea which include 6 Mentha. The Northern part of the country is an important hotspot of the floral diversity and has a collection of many important indigenous medicinal plants that are regularly used by the people living in the surrounding area. Ethnomedicinally, the plant species of lamiaceae which are used by local people of the area includes *Mentha arvensis* and *Mentha piperita*, commonly known as podina, is used as emollient, operient, demulcent, antidiabetic and carminative while *Oreganum vulgare* (Ban ajwain) is used for colic, uterine disorders and epilepsy. *Mentha longifolia* is used as a condiment, carminative and stimulant, remedy for headaches, stomach trouble, digestion, purification of blood and anti-emetic. *Salvia nubicola* is used in treatment of lung diseases and asthma of cattles (Phillipson,1994). Keeping in view impotence of medicinal plants especially *menthe longifolia*, present study was under taken with following aims and objective Extraction of essential oils from plants and chemical analysis and quantification of constituents of essential oils by gas chromatography-mass spectrometry Exploring the anti-microbial activity of essential oils against human pathogenic bacteria.

MATERIAL AND METHODS

Collection of samples

Fresh aerial parts of *Mentha arvensis, Mentha longifolia* and *Mentha spicata* were collected from District Abbottabad. The samples were collected on the basis of ethnobotanical uses of menthe species in folk medicines by inhabitants of this area. The samples were collected in fine plastic bags duly labeled with name, location and area of collection of plant samples. The samples were identified by expert taxonomist at the Department of Environmental Sciences, COMSATS Institute of Information Technology, Abbottabad and voucher specimens (No. 132) were

deposited for future reference. About 3 kg of hand plucked plant materials were packed in plastic bags and shifted to Biochemistry laboratory, Department of Chemistry, COMSATS Institute of Information Technology, Abbottabad. The aerial parts of the *mentha* species were utilized for the extraction of essential oils as well as for determination of other phytochemicals and for assessment of bioactivities.

Preparation of samples

Plant materials were washed with distilled water to remove unwanted materials including dust. Then samples were shade dried followed by sun and oven drying at lower temperature for one night. The dried samples were ground with the help of electric grinder, sieve 80 mesh and stored at 4 °C till further use.

Analysis of plant extracts for phytochemicals

Determination of flavonoids

The total flavonoid content (TFC) was determined by spectrophotometric method (Skehan *et al*., 1990), with some modifications and analysis were carried out in triplicate form. A 100 ppm standard solution of Quercetin was prepared in conical flask by adding 2.5 mg of Quercetin in 25 mL methanol. The TFC of sample was determined by using standard curve made by using six different concentrations (10, 20, 40, 60,80 and 100 ug/mL) of (Sigma-Aldrich). Sample solution (0.5mL) was put in all falcon tubes to which 0.5 mL distilled water and then 0.5 ml solution of $AlCl_3.6H_2O$ was added. Then the falcon tubes were incubated for 5 mins at room temperature. After incubation, 2mL of NaOH solution (1M) was added and mixed well. The absorbance was measured at 510 nm by using spectrophotometer. The total flavonoids were expressed as µg of

quercetin equivalent (QE) per g of sample. Three replicates of each sample was used and average results were calculated as QEg/100g.

Estimation of Total Phenolic Content

Total phenol content (TPC) in plant extracts was generally determined according to Folin-ciocalteu method (Phillipson, 1994). A 100 ppm standard solution of Gallic acid was prepared in methanol (2.5 mg of gallic acid in 25mL methanol) in a conical flask. The TPC of sample was determined by standard curve made by using different concentrations (10, 20, 40, 60, 80 and 100 µg/mL) of gallic acid (Sigma-Aldrich). 0.5 mL sample solution and 0.5 ml distilled water was mixed with 1ml of 10 % Folin-Ciocalteu's phenol reagent. After incubation for 5 mins at room temperature, 2mL of sodium carbonate solution was added and was then mixed well. Then the mixture was incubated at room temperature in dark for 90 mins. After incubation, the absorbance of solution was recorded at 760 nm versus the blank on UV-Visible spectrophotometer. Three replicates of each sample were taken and final results were expressed as standard Gallic acid equivalent (Gulfraz *et al*., 2008).

Determination of Saponins

Saponin content from three plant samples was determined according to the method described by (Skehan *et al*.,1990). The 20g of each plant sample was dispersed in 200 mL of 20% ethanol. The suspension was heated over a hot water bath for 4 hrs with continuous stirring at about 55 °C. The mixture was filtered and the residue was re-extracted with another 200 mL of 20% ethanol. The combined extracts were reduced to 40mL over water bath at about 90 °C. The concentrate was transferred into a 250 mL separatory funnel and 20 mL of the diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ethyl ether was discarded. The purification process was repeated and 60 mL of n-butanol was added. The combined n-butanol extracts were washed twice with 10 mL of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the samples were dried in

the oven to a constant weight and saponin content was calculated in percentage (Schwartmann *et al*., 2002; Sharma *et al*., 1975).

Quantification of tannins

Extraction of tannins was carrie out according to the method reported by (Ullah and Khan, 2008) . A mass of 100g of dried powder of plant was placed in an Erlenmeyer flask with 400mL of 70% acetone. The flask was covered with aluminium foil to avoid light exposure. This mixture was refluxed at 60 °C for 12 hrs. After this process, the sample was filtered using Whatman filter paper no.41 and centrifuged at 3500 rpm for 15 mins. The solvent was removed using a rotary evaporator at a maintained temperature below 60 °C and light exposure was avoided by wrapping the flask in aluminium foil. Quantitative determination of total tannins was carried by Folin-ciocalteu method. In this assay, 800 µL of the sample was put into a test tube and mixed with the same volume of Folin-ciocalteu reagent, shaken and left for 5 mins. The solution was diluted with 5 mL of distilled water and analyzed in a UV-Visible spectrophotometer at 725 nm for the determination of total tannins. The obtained absorbance values were analyzed against the standard curves prepared with tannic acid for total tannins and expressed as mg standard equivalent/g of plant extract.

Extraction of Essential oils

Steam distillation is a separation process used to purify or isolate temperature sensitive materials, like natural aromatic compounds. Steam or water is added to the distillation apparatus to lower the boiling points of the compounds. The basic principle of steam distillation is that it allows a compound or mixture of compounds to be extracted at a temperature considerably below that of the boiling point of the individual constituent. Essential oils contain substances with lower boiling points and in the presence of steam or boiling water, these compounds are volatilized at a temperature of about 100 °C. The essential oil from fresh aerial parts of the plant was extracted. The aerial parts of the selected plants were cut into smaller pieces with the help of knife and scissors. The small pieces of plant material could yield greater biomass that is easily extracted with high efficiency. After converting into small pieces, the weight of aerial parts of plant was

determined by using an electric digital balance. The weighed plant material was then charged into a stain-less vessel of distillation apparatus. Approximately 2000 mL distilled water was added to the vessel and then placed on heating mantle. The vessel was then connected with a condenser that was cooled by cold tap water. The flow of water through a condenser was started and then the resulting mixture was boiled for 3 hrs. The distillate containing volatile compounds was collected in a separating funnel after passing through the water condenser. Layer of oil at the top of water in separating flask clearly revealed the presence of essential oil. Then essential oil floating on distillate was separated and the remaining distillate was used to separate oil from it by liquid-liquid extraction (Upadhyay, 2015).

Liquid-Liquid extraction

Liquid-liquid extraction (solvent extraction) is a separation process which is based on the different distribution of the components to be separated between two liquid phases. As essential oil is a mixture of organic compounds so easily extracted by using organic solvents like hexane, chloroform, ethyl acetate, methanol or acetone etc. But in liquid-liquid extraction from water substrate, only water immiscible solvents could be used which include hexane, ethyl acetate and chloroform etc. In this process 70 mL HPLC grade n-hexane was added to distillate collected in separating funnel. The stopper of separating funnel was opened just after the addition of hexane to reduce the pressure produced due to hydration of hexane. The stopper of funnel was closed and the resulting mixture was shaken gently for some time to dissolve maximum amount of essential oil in hexane. The stopper of separating funnel was opened for some time to release pressure. The shaking process was repeated at least three times and the separating funnel was hanged in a stand holder. After a few minutes, a clear layer of hexane containing essential oil was formed above the water which was separated in another flask. The same procedure was repeated three times by adding 70 mL of hexane in the residual distillates. Some traces of water still present in hexane extract was removed by the addition of small amount of anhydrous magnesium sulphate to hexane extract. Hexane extract containing magnesium sulphate was then filtered in a pre-weighed round bottom flask (Valko *et al*.,2006).

The excess hexane was evaporated by the help of rotary evaporator (Buchi R100) at 25°C under reduced pressure. The hexane free essential oil was weighed on an analytical balance and the

yield (%) of the oil was determined by dividing the extracted oil mass by the mass of plant used for the extraction of oil. Round bottom flask containing hexane extract was connected to rotary evaporator by means of specific glass adopter. A collecting flask was connected to the condenser of rotary evaporator to collect the hexane after evaporation. At the start, the vacuum of rotary evaporator was set at 200 mbar at 25 °C. After 3 mins, when the evaporation from flask stabilized, the pressure was reduced to 100 or 80 mbar. This process was carried out until all the hexane was evaporated from round bottom flask. The pure essential oil was weighed and the yield (%) of the essential oil was determined by dividing the mass of essential oil to the mass of fresh plant material used for the extraction of oil.

Chemical analysis of the essential oil by GC-MS

The chemical composition of the extracted oil was investigated by using Gas Chromatography-Mass Spectrometry (GC-MS). The Hawlett Packard GC-MS system was used to analyze the samples. The Hawlett Packard GC 6890N was equipped with DB-5 column (30 m length, 0.25 mm internal diameter and 0.25 µm stationary phase film thickness) and coupled with a HP 5973 Mass Spectrometer (MS; Agilent Technologies Inc. USA). The injector was operated at 235°C . The oven temperature of GC was programmed as: the initial temperature of oven was 40 °C and it was maintained for 2 min, the temperature was raised from 40 to 450 °C at a heating rate of 4 °C and remained at higher temperature for 8 mins. Highly pure helium gas was used as mobile phase with a constant flow of 1mL/min. The dilute solution of essential oil was injected with volume of 1 µL in split less mode (Upadhyay, 2015).

The FT-IR spectra of defatted samples was recorded in FT-IR instrument (Model 1:1 FS 25, Bruchure, Germany) with PC based software controlled instrument operation and data processing. A small amount of powdered sample made in pellets using KBr for FT-IR analysis and a thin film was prepared by applying pressure. The data of IR transmittance was collected at a wave number ranged from 4000 cm^{-1} to 500 cm^{-1}. All the samples were analysed triplicates with KBr pellets as blank. The spectra were compared with a reference to identify the functional groups existing in samples.

Determination of bioactivities

The antioxidant activity of plant extracts was carried out according to method earlier reported by many authors . Therefore for determination of antioxidants, following bioassays were used.

DPPH scavenging activity was done according to method (Graf *et al*., 2010) with some modifications. This method was based on the ability of antioxidant to scavenge *1, 1- diphenyl-2-picryl hydrazyl* (DPPH) action radical. In a falcon tube, 100 µL of the sample solution was taken and then 4 mL of DPPH solution (0.1 mM) was added and mixture was vortexed vigorously. Then the mixture was incubated with Aluminium foil to avoid heat exposure in the dark for 30 mins at room temperature. *UV- Vis spectrophotometer was used to measure the absorbance of the solution at 517 nm.* Percentage inhibition was calculated by

$$\% \text{ Inhibition} = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100$$

ABTS scavenging bioassay

The ABTS radical scavenging bioassay was carried out. The ABTS radical cation was prepared by mixing 5 mL of a 7 mM aqueous ABTS solution to about 88µL of 140 mM potassium persulphate ($K_2S_2O_8$) by making the final concentration of a solution to be 2.45 mM. The solution was incubated in dark for 16 hours. After incubation, the radical cation was further diluted in ethanol just to make the initial absorbance value of solution to be 0.7. Then the plant extracts to be studied were prepared in ethanol to a volume equal to 25µL. 10µL of sample solution was added to the reaction mixture and the final percentage reduction in absorbance was measured at 730 nm. The expected final absorbance was assumed to be 20-80% decreased as compared to the initial absorbance of reaction mixture (Wall *et al*., 1966).

Scavenging of H_2O_2

Scavenging of H_2O_2 was determined. About 4 mM H_2O_2 0.6mL solution (prepared in PBS) was added to 4mL solution of extract and incubated for 10 min. The absorbance of the solution was measured at 230 nm against a blank solution, by using spectrophotometer method. Free radical scavenging potency as determined from %age H_2O_2. Lower H_2O_2 indicated strong free radical scavenging activity (Ullah and Khan,2008).

Brine Shrimp toxicity assay

Brine shrimp eggs were hatched in a shallow rectangular dish (22 × 30 cm) filled with artificial sea water which was prepared with a commercial salt mixture and double distilled water. A plastic divider with 2 mm holes was clamped in the dish to make two unequal compartments, the eggs (50 mg) were sprinkled into the larger compartment which was darkened while the smaller

compartment was illuminated. After 48 hrs, the phototropic napulii were collected by pipette from the illuminated side, which was separated by the divider from their shells. Twenty shrimps were transferred to each sample vial using pipette and 5 mL artificial seawater was added. The napulii can be counted in the stem of pipette against a lighted background. A drop of dry yeast suspension (3 mg in 6 mL artificial seawater) was added as food to each vial. The vials were maintained under illumination. Survivors were counted with the aid of 3 magnifying glasss and after 24 hrs percent death at each dose and control were determined. In each case where control deaths occurred, the data were corrected using Abbott's formula:

% death = [Test – Control] / Control × 100

Determination of Antibacterial activity

Suspension of organisms was prepared as per McFarland's standard. A 24 hours old culture was used for the preparation of bacterial suspension. Bacterial strains (colony) were picked in eppendorf tubes with the help of *inoculation loops* and placed in 37°C incubator for 30 minutes. Each bacterial colony collected was then mixed in 3 mL distilled water and then shaked vigorously or vortexed and optical density was checked and made equal to 0.5 by using spectrophotometer at 600 nm wavelength.

Antibacterial activity of mentha essential oil

Antibacterial activity of essential oils extracted from M. arvensis, M. longifolia and M.spicata was assessed using agar well diffusion method (Upadhyay, 2015) . Nutrient agar was prepared by pouring accurately weighed 13g/L of Nutrient broth and 14 g/L of agar technical in 1L of distilled water in reagent bottle and was mixed thoroughly so that it may get mixed. Then the mixture was autoclaved *with the temperature at 110 °C for 60 mins. Then* agar plates were poured by 30-35 mLof autoclaved nutrient media, covered and sealed with parafilm. The *covered plates were allowed to rest for an hour so the agar can solidify* and then petri plates were placed in an incubator at 37^{o} C for overnight. 200 µL of each bacterial strain was evenly spread on surface of petri plates using glass spreader. Then four uniform and equidistant wells were made with 6 mm (diameter) cork borer in each plate. Each well was filled with 30µL of essential oil

test solution and 2 replicates of each test petri plate were made. Hexane was used as negative control and streptomycin (1 mg / ml) was selected for positive control as the reference for all bacterial strains. After half an hour, the plates were placed in incubator at 37°C for 24 hours. The size of zone of inhibition of each well in a petri plate was individually measured in millimeters *by using a scale at four different places around the inhibition zone circle* (Wall et al., 1966).

The biological activity tests were conducted by using different concentration ranging from 1-5%. For the biological activities the essential oils solutions were prepared in absolute ethanol. For the preparation of 5% solution, about 1mL of ethanol was added for each 50 mg of essential oil (50 mg/mL). The same procedure was continued for 1% solution in ethanol (10 mg/mL).

Statistical analysis

The bioassay results are presented as an average of four to five replicates data. In way of finding the statistical difference between different treatments, the data obtained were analyzed by using one way ANOVA (analysis of variance).

RESULTS

Analysis of Phytochemicals

Quantitative analysis of extracts indicates the percentage of flavonoids, phenolics, saponins and tanins (Table 1). Result indicated that the aerial parts of *M. spicata* contained the higher amount of flavonoids (15.05 mg/g) followed by *M. longifolia* which contain 14.17mg/g of flavonoids while M. arvensis has 12.21mg/g of flavonoid content in plant extracts. The percentage mg per gram of sample extract of total phenolics was 20.32, 25.149 and 27.637 in *M. arvensis*, *M. longifoilia* and *M. spicata* respectively. The amount of saponins was maximum in plant extract of M. spicata (0.134 mg/g) whereas *M. arvensis* and *M. longifolia* contained 0.04 and 0.01 mg/g of saponins. Similarly the yield obtained of tannins was significant in *M. spicata* (3.51 mg/g) followed by 3.14 mg/g in *M. longifolia* extract while 2.31 mg/g of tannins was found in extract of *M. arvensis*.

Table 1. Quantitative analysis of phytochemicals

Plant name	Flavonoids (mg/g)	Total phenolics (mg/g)	Saponins (mg/g)	Tannin (mg/g)
M. arvensis	12.21±0.07	20.32±0.09	0.040±1.3	2.31±1.5
M. longifolia	14.17±0.07	25.149±0.06	0.013±1.2	3.14±0.9
M. spicata	15.05±0.14	27.637±0.06	0.134±1.2	3.51±0.8

Mean ± Standard Deviation (n=3)

Assessment of yield of essential oils

Essential oils were extracted by hydro distillation from fresh aerial parts of *Mentha arvensis, Mentha longifolia and Mentha spicata.* Fresh aerial parts of *M. arvensis* and *M. longifolia* were rich in essential oil and yielded 1.70% and 1.47% essential oil respectively. M. spicata also showed good yield however, the amount was less as compared with other mentha species (Table 2).

Table 2. Percentage yield of essential oils

Latin name	Family	%age yield of essential oils
Mentha arvensis	Lamiaceae	1.70±0.11
Mentha longifolia	Lamiaceae	1.47±0.31
Mentha spicata	Lamiaceae	1.20±0.03

Mean ± Standard Deviation (n=3)

Chemical analysis of essential oils of *mentha* with GC-MS

The chemical constituents of *mentha* oils were obtained by analysis of oil by GC-MS and results are presented in Tables 3 and 4. In the essential oil of *M. arvensis*, seven main compounds were identified representing (99.1%) of the essential oil. The most abundant compounds were carvone (84.3%) and eucalyptol(5.3%). *M. longifolia* essential oil contained six major compounds representing 98.2% of the oil. Major compounds were piperitone oxide (54.2%), menthone (9.7%), 2-Hydroxy-3-(3-methyl-2-

butenyl)-3-cyclopenten-1-one (8.4%)**,** borneol (4.6%), pulegone (4%) and menthol (3.3%). In *M. spicata* essential oil, four major compounds were identified constituting 96.5% of the oil. The major compounds were 2-Hydroxy-3-(3-methyl-2-butenyl)-3-cyclopenten-1-one (47.1%), eucalyptol (12.0%), α-pinene (9.7%) and borneol (9.5%).

Table 3. Chemical composition of *M. arvensis* essential oil based on total ion chromatogram of GC-MS

S. No	Compound name	RI	RT	%age
1	α-Pinene	930	10.81	0.1
2	Sabinene	970	12.23	0.1
3	β-Pinene	973	12.34	0.1
4	β-Myrcene	988	12.86	0.1
5	3-Octanol	991	12.99	0.1
6	Limonene	1026	14.25	3.2
7	1,8-Cineol	1028	14.33	5.3
8	β-cis-Ocimene	1035	14.58	0.1
9	Cis-Sabinene hydrate	1064	15.66	0.1
10	Linalool	1096	16.86	0.3
11	Trans-p-mentha-2,8-dienol	1118	17.63	0.1
12	Trans-Limonene oxide	1135	18.27	0.1
13	Borneol	1164	19.33	0.7
14	4-Terpineol	1175	19.73	0.1
15	α-Terpineol	1188	20.21	0.4
16	Dihydrocarveol	1193	20.38	0.1
17	Cis-Dihydrocarvone	1195	20.43	0.4
18	Cis-Carveol	1231	21.69	0.3
19	Carvone	1246	22.22	84.3
20	Isopiperitenone	1269	23.03	0.3
21	Piperitenone	1339	25.39	0.2
22	β-Bourbenone	1386	26.94	0.4
23	Jasmone	1396	27.27	0.5

24	Caryophyllene	1422	28.07	0.6
25	β-Farnesene	1455	29.1	0.1
26	Germacrene-D	1483	29.97	0.7
27	Elixene	1499	30.46	0.1
28	Caryophyllene oxide	1586	33.04	0.1
	Total % Identified compounds			**99.1**
25	Piperitenone	1339	25.39	0.2
26	1-Cyclohexene-1-methanol, 4-(1-methylethenyl)-, acetate	1345	25.57	0.1
27	2-Hydroxy-3-(3-methyl-2-butenyl)-3-cyclopenten-1-one	1365	26.25	8.4
28	β-Bourbenone	1386	26.94	0.1
29	Caryophyllene	1422	28.07	1.7
30	Germacrene-D	1483	29.97	0.7
31	Caryophyllene oxide	1586	33.04	0.3
	Total % Identified compounds			**98.2**

Chemical Constituents of *mentha* oils

The identified compounds were listed according to their elution order on DB-5 GC column. Carvone (84.3%), piperitone oxide (54.2%) and 2-Hydroxy-3-(3-methyl-2-butenyl)-3-cyclopenten-1-one (47.1%) are the three major compounds present in *M. arvensis, M. longifolia* and *M. spicata* respectively. Similarly some compounds were found common in plants. For example, eucalyptol was present in higher concentration in *M. spicata* (12%) followed by (5.3%) in *M. arvensis* while it was lowest (0.4%) in *M. longifolia* (Table 3-5). In the same way the concentration of germacrene-D was (2.5%) in *M. arvensis* followed by (0.7%) in *M. longifolia* and *M.spicata* each. Borneol was (9.5%) in *M. spicata* while (4.6%) in *M. longifolia*. Caryophyllene was also common in all the plants though its concentration varied in each plant that is (0.6%), (1.7%) and (1.3%) in *M. arvensis*, *M. longifolia* and *M. spicata* respectively.

Analysis of extracts with FT-IR

Fourier Transform Infrared Spectroscopy (FT-IR) identifies chemical bonds in a molecule producing a profile of the sample, a distinctive molecule fingerprint that can be used to screen and scan samples for many different components. FT-IR is an effective analytical technique for detecting the functional groups and characterizing covalent bonds.

Table 4. FT-IR analysis of methanolic extracts of *mentha* species

Sr. No	Wavelength	Bond	Functional Group
1	3337.45	O-H, H-bond	Alcohol, Phenol
2	2924.51	C-H stretch	Alkanes
3	1710.21	C=O stretch	Carboxylic acids, Carbonyls
4	1514.05	CO asymmetric stretch	Nitro compounds
5	1451.07	C-C stretch	Aromatic
6	1030.75	C-O	Alcohols, Esters, Ethers
7	817.84	C-Cl stretch	Alkyl halides
8	725.27	C-Cl stretch	Alkyl halides
9	632.43	C-Br stretch	Alkyl halides

Antioxidant activities

DPPH and ABTS scavenging potential

For the evaluation of antioxidant activities of plant extracts of M. arvensis, M. longifolia and M. spicata, the DPPH and ABTS scavenging bioassays were employed. The free radical scavenging activity of the plant extracts was much enhanced in ABTS scavenging assay as compared to DPPH scavenging assay (Tables 5 to 6).

Table 5. Scavenging potential of DPPH

	DPPH Scavenging activity			
Plant extracts Conc. µg/mL	***M. arvensis***	***M. longifolia***	***M. spicata***	***Ascorbic acid***
50	60.7±1.07	70.33±0.4	80.1±0.8	49.29±0.5
100	55.45±1.9	63.77±0.6	73.24±0.62	41.34±1.3
150	48.9±0.13	51.81±0.5	61.33±0.3	33.41±1.7
200	33.62±0.36	36.18±1.0	49.67±0.03	28.67±1.03
250	21.8±0.33	25.42±1.01	33.33±0.2	21.8±1.01
300	19.9±0.33	14.33±2.1	21.9±0.7	13.55±1.23

Table 6. Scavenging potential of ABTS

	ABTS Scavenging activity			
Plant extracts Conc. µg/mL	***M. arvensis***	***M. longifolia***	***M. spicata***	***Ascorbic acid***
50	67.7±1.23	78.73±0.5	88.1±0.9	67.29±0.9
100	59.42±1.8	65.45±0.9	78.44±0.7	56.76±1.09
150	51.89±0.45	58.21±0.7	63.81±0.8	49.87±1.8
200	44.32±0.23	47.81±1.1	55.54±0.01	40.54±1.08
250	38.61±0.10	36.72±1.01	49.01±0.3	34.6±1.1
300	33.62±0.33	29.88±2.1	35.62±0.7	22.55±1.23

H_2O_2 Scavenging potential

The extracts of *M. arvensis*, *M. longifolia* and *M. spicata* significantly scavenged H_2O_2 by 34.9%, 29.66% and 37.9% respectively at concentration of 300 µg/mL (Table 7). *M. arvensis*, *M. longifolia* and *M. spicata* extracts exhibited significant scavenging activity for H_2O_2 on dose dependent manner as shown in table 9.

Table 7 . Scavenging potential of hydrogen peroxide (H_2O_2) by plant extracts

	Percentage of H_2O_2 (%)			
Plant extracts Conc. µg/mL	***M. arvensis***	***M. longifolia***	***M. spicata***	***Ascorbic acid***
50	92.7±1.7	96.46±2.3	87.1±0.6	64.29±0.9
100	90.45±0.9	91.06±0.9	79.05±0.88	52.07±1.2
150	76.9±0.13	83.33±0.77	78.98±0.1	33.41±1.44
200	69.77±0.56	67.11±5.1	67.01±0.05	31.05±1.08
250	51.8±0.45	52.01±1.13	49.34±0.7	24.8±1.1
300	34.9±0.34	29.66±3.22	37.9±0.1	20.45±1.01

Mean±S.D after triplicate analysis

Determination of antibacterial activity

Antibacterial activity of essential oil of *M. arvensis, M. longifolia* and *M. spicata was tested against* the strains of Gram+ bacteria such as *Staphylococcus aureus(KX262674)* and *Bacillus cereus(KX262674*) ;and Gram – bacteria *Escherichia coli (ATCC 10536), Salmonella typhi (ATCC 6539)* and *Pseudomonas aeruginosa (ATCC 9027*). The results indicate that essential oils at different concentrations exhibit significant antibacterial activity. *M. arvensis* showed best antibacterial activity against *S. aureus*, *B. cereus* and *S. typhi* comparable to standard drug used, streptomycine, at concentrations of 100 µg/mL and 200 µg/mL with inhibition zones (9.87, 11.31), (9.36, 11.43) and (6.78, 10.87) respectively in mm. Similarly *M. longifolia* also showed markable antibacterial activity against *B. cereus* with inhibition zones 8.06 mm and 8.25mm at 100µg/mL and 200 µg/mL concentration respectively whereas at conc. 200 µg/mL the inhibition zone of 12.16 mm was measured against *S. typhi*. The essential oil of *M. spicata* showed very good antibacterial activity against *S. aureus* revealing inhibition zones of 8.18 mm and 9.34 mm at 100 µg/mL and 200 µg/mL concentration.

Table 8. Antibacterial activity of essential oils

Sample	**Zone of inhibition (mm)**				
Essential oil	*P. aeruginosa*	*S. aureus*	*B. cereus*	*E. coli*	*S. typhi*
M. arvensis	2.56±0.23	4.5±0.25	4.43±0.23	2.75±0.20	0.87±0.43
M. longifolia	0	3.25±0.16	3.62±0.32	0	3.12±0.43
M. spicata	3.84±0.31	4.75±0.20	3±0.23	1.75±0.5	0
Streptomycine	9.82±0.43	11.65±0.51	11.07±0.32	9.90±0.25	10.79±0.63
n-hexane	0	0	0	0	0

Table 11. Antibacterial activity of essential oils at

Sample	Zone of inhibition (mm)				
Essential oil	*P. aeruginosa*	*S. aureus*	*B. cereus*	*E. coli*	*S. typhi*
M. arvensis	4.75±0.20	6.25±0.30	6.31±0.47	3.56±0.12	3.37±0.14
M. longifolia	0	5.43±0.23	7.18±0.23	2.18±0.12	5.68±0.12
M. spicata	5±0.88	6.18±0.32	4.62±0.14	2.62±0.25	0
Streptomycine	±	10.18±0.23	13.18±0.37	10.18±0.12	±
n-hexane	0	0	0	0	0

Determination of cytotoxicity

The brine shrimp cytotoxicity of extracts of *M. arvensis, M. longifolia and M. spicata* was carried out which indicates that the cytotoxicity of extracts increased by increasing concentration of plant extracts, however overall extracts has shown less cytotoxicity.

Table 9. Brine shrimp assay of plant extracts

Sample name	Conc. (µg/mL)	Total napuli	% of death after 24 hours	
			Live	Dead
M. arvensis	10	20	18	2
	100	20	15	5
	300	18	11	7
	600	19	10	9
M. longifolia	10	18	17	1
	100	20	14	6
	300	20	12	8
	600	19	10	9
M. spicata	10	20	20	0
	100	19	17	2
	300	18	13	5
	600	18	9	9

DISCUSSION

Estimation of phytochemicals

Phytochemicals present in the plants may be used as food and medicine. There is growing worldwide interest for characterization, isolation, the *in vivo* and *in vitro* assessment of biological activities of the phytochemicals to have beneficial therapeutic capacity for curing human from various illness. Therefore, variety of chemical compounds obtained by the tested plants impart significant anti proliferative, cytotoxic, antibacterial and anti-tuberculosis activities. In the present study, the plant extracts were assessed for the presence of flavonoids, total phenolics, saponin and tannin. The study indicates that the presence of these phytochemicals in all the three plants. *M. spicata* extract contained the considerable amount of flavonoids, phenolics and tannins (Flowler, 2006; Graf *et al*., 2010; Koehn and Carter, 2005). .Flavonoids are polyphenolic molecules containing 15 carbon atoms and are soluble in water. Flavonoids are a group of plants metabolites thought to provide health benefits through cell signaling pathways and antioxidant effects (Newan and Cragg, 2012). Phenols constitute the largest group of secondary metabolites, varying in size from a simple structure with aromatic ring to complex ones. Phenolic compounds, ubiquitous in plants are anssential part of human diet, and are of considerable interest due to their antioxidant properties. Saponins are class of chemical compounds found in various plant species. Furthermore saponins glycosides have many health benefits of Tannins (flavonoids) are astringent polyphenolic biomolecules that binds to and precipitates proteins and various organic compounds including aminoacids and alkaloids (Rios, 2010, Packer *et al*.,2004).

Phytochemical prospection of *M. arvensis* extract indicated the presence of different secondary metabolites (Koehn and Carter, 2005). So the presence of significant amount of these important phyto-constituents bestow the plant with high medicinal activities like free radical scavenging and antimicrobial activities (Marles and Farnsworth, 1995).

Chemical composition of Mentha spp

By comparison of chromatogram of GC-MS, the area of peaks of compounds was calculated and other required information was obtained by NIST library data base. The prominent peaks in the chromatogram of essential oil of *M. arvensis* indicated the presence of carvone (84.3%), eucalyptol (5.3%) and limonene (3.2%) whi le other chemical compounds were present in lower quantities. It was reported in literature that experiment conducted in Brazil for analysis of *M. arvensis* and GC-MS indicated a significant amount of menthone (69.77%) while p-Mentone (12.00%) have shown an excellent antibacterial activity While another reported data indicates the presence of Menthol (78.90%) and isomenthone (6.35%) in the essential oil of M. arvensis (Hussan *et al*., 2014).

The chemical composition of essential oil of M. longifolia contains the major compounds to be piperitone oxide (54.2%), menthone (9.7%), 2-Hydroxy-3-(3-methyl-2-butenyl)-3-cyclopenten-1-one (8.4%), Borneol (4.6%), Pulegone(4%), 1,3,3-Trimethyl-2-oxabicyclo[2.2.2]octan-6-one (3.6%) and Caryophyllene (1.7%). Similarly, the GC-MS analysis of chemical compounds in M. spicata revealed that the major compound found in its essential oil was 2-Hydroxy-3-(3-methyl-2-butenyl)-3-cyclopenten-1-one (47.1%). 1,8-cineol (12.0%), α-Pinene (9.7%), Borneol (9.5%), Germacrene-D (2.5%), β-Pinene (1.7%), β-Myrcene (1.6%), Caryophyllene (1.3%) and Linalool (1%) were also present in the essential oil. The percentage of 1,8-cineol was found to be 6.36%, 1,8-Cineol 4.7% and 14.5% in essential oil of *M. spicata*[173], [116], [106]. Caryophyllene has been reported in India to be 2.35% in the essential oil [94]. α-Terpineol has been reported in its essential oil as well (Rios, 2010).

The variation in chemical constituents of essential oils might be due to the variation in the *mentha* species when cultivated. The composition of *mentha* essential oil directly affects the effectiveness of biological

activities which have displaces difference in its constituents depending on the growing area whereas chemistry of *mentha* oil is complex and variable as mentioned above. The seasonal changes may account in the variation in chemical constituents of plants grown at different parts of the world .Chemical constituents with antioxidant activity present in plants determine the role of plants in prevention of many degenerative diseases. The human food suppliments including herbs, contain higher amount of compounds that are capable of deactivating free radicals . The plant extracts were assessed for their antioxidant potential by three methods that includes DPPH scavenging, ABTS scavenging and H_2O_2 scavenging bioassays. The methanolic extracts of the plants showed significant scavenging of free radicals. By comparing DPPH and ABTS bioassays results. It was assumed that the antioxidant potential of the three plant extracts is much increased in case of DPPH bioassay as compared to ABTS bioassay. Among the three plants, the antioxidant potentialof *M. longifolia* was very significant followed by *M. arvensis* and the extract of *M. spicata* be the least in antioxidant potential as compared to other tested extracts (Rios, 2010).

Similarly in the current research work, *M. arvensis* has shown a considerable amount of antioxidant potential. However the studies conducted in Malaysia and Bangladesh reavealed the good antioxidant potential and anti-inflammatory activity of *M. arvensis*. *M. spicata* also possess the antioxidant potential but less as compared to other two plant extracts (Packer *et al*., 2004).

Scavenging of H_2O_2 was exhibited by the plant extracts was dose dependent. The total radical scavenging activity (superoxide and hydroxyl radical) of whole plant extract of *M. spicata* was found to be quite significant and showed effectively in dose dependent manner and suppressed the production of H_2O_2 (37%) at the dose concentrartion 300 µg/mL which is a characteristic of chain-breaking antioxidants, and has been observed in oxidation of linoleic acid emulsion with extract (Upadhyay, 2015).

Flavonoids, phenolic acids and phenolic diterpenes are the examples of phenolic components with antioxidant properties. Scavenging of extracts may be attributed to their phenolic constituents which can donate electron to H_2O_2, thus neutralizing to water. Although H_2O_2 is not very reactive, it can sometime cause cytotoxicity by rising hydroxyl radicals in the cells. Removal of H_2O_2 by food systems is very important (Valko *et al*., 2006).

Antimicrobial activity of essential oil of *M. arvensis, M. longifolia* and *M. spicata* in hexane has been tested against Gram +ve and Gram –ve bacterial strains. The selected herbs have been used traditionally as a remedy for respiratory diseases like bronchitis, sinusitis, tuberculosis and common cold. These activities are mostly due to presence of oils like menthol, carvone, limonene etc. The results indicate that essential oils exhibit significant antibacterial activity. However, essential oil of *M. arvensis* showed much activity against *S. aureus* and *B. cereus* at concentration 100µg/mL and 200µg/mL as compared to other tested bacterial strains while its antibacterial activity against *S.typhi* at 200 µg/mL was also higher (Upadhyay, 2015).

The antibacterial study indicates that the plant essential oils have tremendous antibacterial activity at higher concentrations against various bacterial strains that are the major causative

agents mainly of stomach problems. The finding of zone of inhibition was found to be higher than the results reported by other authors .

Cytotoxicity refers to the ability of certain chemicals to destroy the living cells in the body. By a cytotoxic compound, healthy living cells either induce necrosis (accidental cell death) or apoptosis (programmed cell death). Brine shrimp lethality bioassay used for *M. arvensis, M. longifolia* and *M. spicata* indicates that all the plant extracts are very less toxic and are suitable to be used in folk medicine (Skehan *et al*., 1990) which also indicates importance of *mentha* extracts for its application in pharmaceutical industry for development of drugs.

CONCLUSION

The selected mentha species constitutes a considerable amount of phytochemicals including flavonoids, phenolics, annins and saponins. The major compounds of M. arvensis essential oil were carvone (84.3%), 1,8-cineol (5.3%) and limonene (3.2%) while major compounds of M. longifolia were piperitone oxide (54.2%), menthone (9.7%), 2-Hydroxy-3-(3-methyl-2-butenyl)-3-cyclopenten-1-one (8.4%), borneol (4.6%) and menthol (3.3%). 2-Hydroxy-3-(3-methyl-2-butenyl)-3-cyclopenten-1-one (47.1%), 1,8-cineol (12%), *α-pinene* (9.7%), borneol (9.5%) and germacrene-D (2.5%). The organic compounds obtained by GC-MS are likely to be the main antioxidants.

The antibacterial study indicates that plant essential oils showed better activities at higher concentration against tested strains. The zone of inhibition were found to be much relatable to the reported data. Brine shrimp lethality bioassay indicates that these plants are non-toxic and are suitable for use as folk medicine and may be used in the preparation of drugs. Essential oils of M. arvensis and M. longifolia are a potent source of mosquito repellents.

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