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Ankita Sharma
School of Studies in Microbiology, Jiwaji University, Gwalior, Madhya Pradesh, India

Sapan Patel
School of Studies in Botany, Jiwaji University, Gwalior, Madhya Pradesh, India

Correspondence
Ankita Sharma
School of Studies in Microbiology, Jiwaji University, Gwalior, Madhya Pradesh, India

Preliminary phytochemical analysis and antibacterial activity of *Mentha arvensis* L. against *Xanthomonas citri*

Ankita Sharma and Sapan Patel

Abstract
The main aim of present investigation was to evaluate the preliminary phytochemical analysis and antibacterial activity of *Mentha arvensis* against *Xanthomonas citri*. *Mentha arvensis* commonly known as "Pudina" in Hindi, "Corn mint" or "Field mint" in English and in Sanskrit it is also known as "Rochani". In this study we have observed some effective results of methanolic extract of *Mentha arvensis* against *Xanthomonas citri*. The results of inhibitory activity of methanolic plant extract compare with the standard antibiotic Kanamycin. The results of zone of inhibition of the extract of *Mentha arvensis* at the concentration of 100 µl was 29.5 mm (leaves) and the zone of inhibition of the standard antibiotic Kanamycin was 32 mm. Hence, the result of *Mentha arvensis* (leaves) is so closer to the standard antibiotic. Therefore, there is a scope to use *Mentha arvensis* (leaves) extract against *Xanthomonas citri* as bactericides. In this study we have also screened active components or secondary metabolites of *Mentha arvensis* through the process of preliminary phytochemical analysis and the result showed the presence of various active components like alkaloids, tannins, steroids, saponins, terpenoids.

Keywords: Antibacterial activity, *M. arvensis*, *X. citri*, Phytochemical analysis

1. Introduction
Crop loss is one of the major problems in all over the world due to plant disease caused by plant pathogenic bacteria, fungi, viruses and insects/ nematodes. In India most of the plant diseases were caused by bacteria. Different diseases of plants are caused by different genera like – *Pseudomonas, Xanthomonas, Xylella, Xylophillus, Acidovorax, Agrobacterium, Erwinia*, *Pantoea, Ralstonia, Burkholderia, Clavibacter, Streptomyces, Spiroplasma* and *Phytoplasma* (Ellis *et al*. 2008) [5]. Among different genera, *Xanthomonas* is a very important kind of plant pathogenic bacteria, which is one of the main causal organisms of different diseases on crops, fruits and vegetables in all around the world. It is a Gram- negative bacterium. Its cellular structure is flagellated and rod-shaped. It belongs to family Xanthomonadaceae. Pathovars of *Xanthomonas* are known to cause disease on several vegetable and cash crop and are reported to have developed resistance to Kanamycin, Ampicillin, Penicillin and Streptomycin (Nafade and Verma, 1985. Rodriguez *et al.* 1997) [12, 15]. *Xanthomonas citri* is a plant pathogenic bacterium, which is responsible for citrus canker, a disease which results in heavy economic losses to the citrus industry as well as farmers. The distribution of citrus canker occurs in Asia, South America, The United States, parts of Oceania and some islands of the African continent. The symptoms of *X. citri* on infectious plant is lesions appear on leaves, twigs and fruit which cause defoliation, premature fruit abscission and blemished fruit and can ultimately destroy the plant. There have been different methods for control of bacterial diseases in plants by the use of different methods like spraying with antibiotics and copper compounds along with pesticides are usually applied. Microorganisms have developed resistance against antibiotics and this has created risk in the treatment of infectious diseases and phytopathogens. These synthetic pesticides cause harmful effect to the environment and also provide direct effect to human as well. Recently, in plant pathology the great attention is dedicated to search and produce medicinal plant extracts. This is one of the best ways to substitute synthetic pesticides and antibiotics for inhibition of plant pathogens and also provide beneficial effect to environment and

living being in the world. Mother earth has gifted us lots of medicinal plants having ability to cure the ailment of human beings as well as plants. *Mentha arvensis* commonly known as "Pudina" in Hindi, "Corn mint" or "Field mint" and in Sanskrit it is known as "Rochani". Wild mint is an herbaceous perennial plant generally growing to 10–60 cm (3.9–23.6 in) and rarely up to 100 cm (39 in) tall. *Mentha arvensis* is a species of mint with a circumboreal distribution. It is native to the temperate region of Europe, Western and central Asia, east to the Himalaya, eastern Siberia and North America. *Mentha arvensis* leaf and oil contain acetaldehyde, amyl alcohol, methyl esters, limonene, β-pinene, β- phellandrene, cadinene, dimethyl sulphide, traces of α- pinene, sabinene, terpinoline, trans-ocimene, g-terpinene, fenchene, α-thujone, β- thujone, citronellol and lutiolin- 7-o-rutioside. It is also include menthol (35-70%), menthone (15-30%), menthyl acetate (4-14%) and pulegone (1-4%). All plants synthesis chemical compounds which are classified based on their chemical class, biosynthetic origin and functional groups into primary and secondary metabolites. Primary metabolites are directly involved in growth and development such as sugar and fats and secondary metabolite are not involved directly and they have been worked as biocatalyst (Geetha and Geetha, 2014) [7]. Secondary metabolite, which are biosynthesized in a smaller range of plants, serving a more specific function including growth regulation, inter and intra-specific interaction and defence against predators and infections. In recent years, secondary plant metabolites (Phytochemicals) previously with unknown pharmacological activities have been extensively investigated as a source of medicinal agents (Krishnaraju *et al*. 2005) [11]. The antimicrobial activity of plant material typically results from the combination of these metabolites present in the plants such as- alkaloids, steroid, tannins, phenol compound, flavanoid and essential oils which are synthesized and deposited in specific parts or in all parts of plant. These plant based natural constituents can be derived from the any part of the plant like- Stem, Leaves, Root, Bark, Flowers, Fruits and Seeds etc. (Gorden *et al.* 2001) [6]. These are generally obtained from plant material by steam distillation or by extraction process with organic or aqueous solvents. Thus, the objective of present study is to determine the phytochemical analysis and antibacterial activity of methanolic extract of leaves and stem of *Mentha arvensis* against *Xanthomonas citri.*

Materials and Methods

Collection of Plant material

Different parts of plants like leaves and stem were collected from Jiwaji University campus, Gwalior (M.P.), India during the month of February to March 2016.

Preliminary Phytochemical analysis

Qualitative phytochemical analysis for the identification of secondary metabolites was carried out for methanolic extracts. The plant parts (leaves and stem) were shade dried in laboratory and grind into homogenized powder and stored in airtight bottles. Those plant parts were subjected to preliminary or qualitative chemical screening for the identification of various classes of active chemical constituents using standard prescribed methods (Harborne, 1984. Adebayo and Ishola, 2009 Sofowora, 1993. Trease and Evans, 1989) [9, 2, 18, 20].

For certain compounds several tests were carried out. Positive result of any one test was considered as an indicative of the presence of that compound. The reason of this is that certain tests are possibly more sensitive than others. Positive tests was denoted as (+) and absent was (-).
The following active compounds observed in plants are as follow:

1. Alkaloids.
2. Tannins.
3. Steroids.
4. Flavanoid.
5. Anthraquinones.
6. Phlobatannins.
7. Glycosides.
8. Saponins.
9. Terpenoids.

Alkaloids

Powdered extract was warmed with 1% aqueous hydrochloric acid for two minutes. The mixtures were filtered and few drops of Dragendroff's reagent were added. A reddish- brown color and turbidity with the reagent indicated the presence of Alkaloids.

Flavonoid

Small quantities of the extracts were dissolving in 10% of sodium hydroxide (NaOH) and Hydrochloric acid (HCl). A yellow solution that turned colorless on addition of HCl indicated the presence of flavonoids.

Anthraquinones

Powdered extracts was shaken with 10 mL of benzene. The solution was filtered and 5 mL of 10 % NH_4OH solution was added to the filtrate. A pink, red or violet color in the ammonical (lower) phase indicated the presence of anthraquinones.

Glycosides (Borntrger's test)

Crude extract was mixed with 2 mL of dilute sulphuric acid and 2 mL of 5 % aqueous ferric chloride solution, boiled for 5 minutes which lead to oxidation to anthraquinones, indicating the presence of glycosides.

Tannins

Powdered extract was stirred with 10 mL of hot distilled water, filtered and ferric chloride was added to the filtrate and observed for blue- black, blue-green or green precipitate.

Steroids (Salkowski test)

Crude extract was mixed with chloroform and a few drops of conc. H_2SO_4, shaked well and allowed to stand for some time. Red color appeared at the lower layer indicated the presence of Steroids.

Saponins (Frothing test and Emulsion test)

Small quantity of powdered extract was boiled in 10 mL of distilled water for 5 minute and decanted while still hot. The filtrate was used for the following test.

(a) Frothing test 1 mL of filtrate was diluted with 4 mL of distilled water and mixture was shaken vigorously and observed for persistent foam which lasted for at least 15 minutes.

(b) Emulsion test This was performed by adding 2 drops of olive oil to the frothing solution and shaken vigorously. Formation of an emulsion indicated a positive test.

Phlobatannins
Deposition of a red precipitate when an aqueous extract was boiled with 1% aqueous hydrochloric acid indicated the presence of phlobatannins.

Terpenoids
5 mL of aqueous extract of each plant sample is mixed with 2 mL of $CHCl_3$ in a test tube 3 mL of concentrated H_2SO_4 is carefully added to the mixture to form a layer. An interface with a reddish brown coloration is formed if terpenoids constituent is present.

Preparation of Plant Extract
Fresh leaves and stem of *M. arvensis* were washed 2-3 times with tap water and subjected to shade drying at room temperature. The dried plant material was powdered using a clean mixer grinder and filled in air tight container and store in a dry place on room temperature until analysis (Harborne, 1979) [8].

Methanol Extraction
The powdered materials of *M. arvensis* were extracted with methanol. During extraction the ratio was taken 1:10 placed into Soxhlet apparatus which run for ten cycles. The duration of each cycle was about 55 minutes. After the completion of ten cycles the colour of powdered material was disappeared or light. After extraction the crude extract were evaporated at 40 ^{0}C with the help of Hot plate stirrer. The extracts were collected and stored at 4^0 C in sterile air tight containers for further analysis (Harborne, 1979) [8].

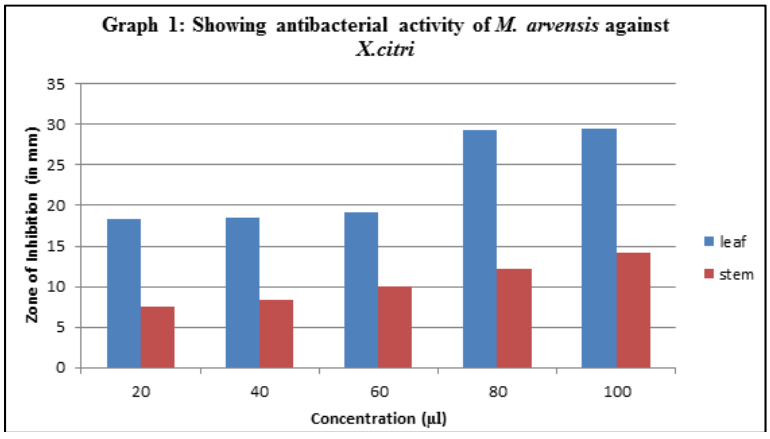
Antibacterial assay
Xanthomonas citri (ITCC NO. BN0001) was procured from the Indian Agriculture Research Institute (IARI), Pusa, New-Delhi. The antibacterial activity of methanol extract of leaves and stem of *M. arvensis* was tested by agar well diffusion method (Akpata and Akinrimisi, 1977) [1]. The extract of leaves and stem of *M. arvensis* were dissolved in DMSO (Dimethyl Sulphoxide) in a concentration of 100mg/ml. In this method wells were made in Muller Hinton Agar (MHA) medium using sterile cork borer after the spreading of bacteria. The method is suitable for organisms that grow rapidly overnight at 35-37 ^{0}C. The previously inoculated bacterial strain was spread on MHA. After few minute, five wells were made in each Petri plate and loaded with different concentration (20, 40, 60, 80 and 100µl). Similar concentration (20, 40, 60, 80 and 100 µl) of antibiotic Kanamycin solution was added in another plate for positive control. Plates were incubated at 37 ^{0}C for 24hrs. The zone of inhibition of bacterial growth around each well is measured and the susceptibility is determined. Antibacterial activity was evaluated by measuring zone of inhibition by using Hi-media zone scale.

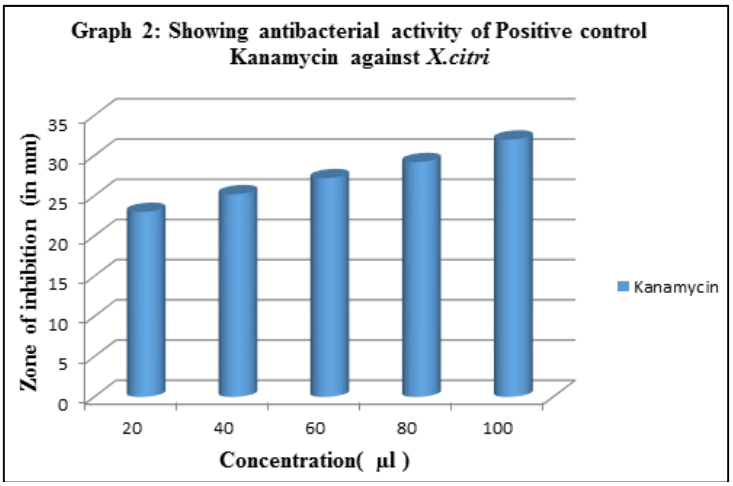
Result and Discussion
The antibacterial activity of Methanolic extract of *M. arvensis* was investigated using agar well diffusion method, against *Xanthomonas citri* at different concentration (20µl, 40µl, 60 µl, 80 µl and 100 µl) after 24 hours of incubation we observed that the zone of inhibition was shown in Table 1:

Table 1: Antibacterial activity of *M. arvensis* (Leaf and Stem) against *Xanthomonas citri* on different concentration compared with positive controls (zone of inhibition in mm)

S. No.	Concentration (µl)	Sample	Methanolic extract (ZOI in mm.)	Kanamycin (ZOI in mm.)
01	20	Leaf	18.3	23.0
		Stem	7.5	
02	40	Leaf	18.5	25.2
		Stem	8.4	
03	60	Leaf	19.2	27.0
		Stem	10.0	
04	80	Leaf	29.3	29.2
		Stem	12.2	
05	100	Leaf	29.5	32.0
		Stem	14.2	

Zone of Inhibition (ZOI)





The antibacterial activity of Methanol extract of *M. arvensis* (leaves) showed the maximum zone of inhibition 29.5mm at the concentration of 100 µl against *X.citri* followed by 29.3mm at 80 µl concentration of *M. arvensis* (leaf), 19.2mm (leaf) at 60 µl, 18.5mm (leaf) at 40 µl, 18.3mm (leaf) at 20 µl, 14.2mm (stem) at 100 µl, 12.2mm (stem) at 80 µl, 10.0mm (stem) at 60 µl, 8.4mm (stem) at 40 µl and 7.5mm (stem) at 20 µl of concentration. Highly significant antibacterial activity was observed in *M. arvensis* (leaf) 29.5mm at the concentration of 100 µl. We have done parallel experiment with Positive control antibiotic Kanamycin which shows significant antibacterial activity against *X. citri,* which are showed in Table -1 and Graph 2.

Herbal medicines are affecting from fringe to mainstream use with a greater number of people seeking remedies and health approaches free from side effects caused by chemicals. Recently, considerable attention has been paid to utilize bio friendly and eco friendly plant based products for the prevention and cure of different plant diseases. India is sitting on a gold mine of well recorded and traditionally well- practised knowledge of herbal medicine. This country is perhaps the largest producer of medicinal herbs and is rightly called the botanical garden of the world. Fresh and healthy leaves and stem part of the plant were collected and shade dried. The leaves and stem were then powdered and sequential extraction was performed using methanol solvent. The antimicrobial properties of many medicinal plants have been previously studied (Rahman, 2004. Nair *et al*. 2005. Joshi *et al*. 2011) [16, 13, 10]. Very little work has been done for the use of *M. arvensis* against *X.citri*. For the comparison, positive and negative controls were used. Negative controls did not show any inhibitory action against test organism, while positive controls significantly inhibit the growth of test organism. The findings match with that of other workers (Bhattacharjee *et al*. 2006) [4]. It is often reported that Gram positive bacteria are more sensitive than Gram negative bacteria to plant based organic extracts (Reynolds, 1996. Benzic *et al*. 2003. Rahman *et al*. 2009) [14, 3, 17]. worked on pharmacognostic standardization, physico and phytochemical evaluation of aerial Parts of *Mentha arvensis* Linn. In the present study we also determine the Preliminary Phytochemical analysis of methanolic extract of medicinal plant *Mentha arvensis* Linn. The investigation showed that the methanolic extract of *Mentha arvensis* contains alkaloids, steroids, saponins, tannins. The results were shown in Table 2.

Table 2: Preliminary Phytochemical analysis of methanol extracts of *Mentha arvensis* Linn for the presence of various phytocompounds

Conclusion

Plat part used	Solvent type	Alkaloids	Flavonoids	Steroids	Tannins	Glycosides	Saponin	Phlobatannins	Anthraquinones	Terpenoid
Leaf	Methanol	+	-	-	+	-	+	_	_	+
Stem	Methanol	+	-	+	+	_	+	_	_	+

In this study we can conclude that the selected leaf and stem extracts of *Mentha arvensis* Linn were showing excellent inhibitory zone against *X.citri* and this happened due to the presence of many secondary metabolites. *X.citri* affected many citrus crop worldwide resulted in the economic loss of farmers every year. This study is an initial step to inhibit the use of chemical pesticides and protect our environment. Phytochemical analysis of methanolic extract of *Mentha arvensis* leaves and stem extracts were obtained by soxhlet method. The screening of phytochemical constituents of *Mentha arvensis* indicated the presence of many secondary metabolites like alkaloids, tannins, steroids, saponins, terpenoids.

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